ACUTE MYELOID LEUKEMIA:
UNDERSTANDING THE GENETIC MECHANISMS

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This dissertation is dedicated to my father, Luis Armando Bottazzi Suárez.

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TABLE OF CONTENTS

ACKNOWLEDGMENTS iii

LIST OF TABLES vii

LIST OF FIGURES viii

ABBREVIATIONS x

ABSTRACT xii

CHAPTERS

1. BACKGROUND AND SIGNIFICANCE 1

- Introduction 1
- Acute Myeloid Leukemia 3
  - Introduction 3
  - Molecular Biology of AML 5
- Oncogenes and Leukemia 7
  - Oncogene Activation 7
  - Oncogene Induced Transformation 14
  - Oncogenes as a Cause of leukemia 14
  - Oncogenes and Human Leukemias 16
- Animal Models for AML 17
  - Introduction 17
  - Avian Leukemia Viruses 20
  - Avian Myeloblastosis Virus 22
  - The BM2/BM2L Model 26

2. MATERIALS AND METHODS 30

- Southern Analysis 30
- Sequencing 31
- RNA Extraction and Northern Blots 32
- Plasmids and Probes 33

Western Analysis 33
Immunofluorescence 34
Cell Lines and Growth Media 34
In Vivo Experiments 35
Differentiation Assays 36
Electroporation 37
Somatic Cell Fusions 37
Differential Display 38
Retroviral Infections 39
Genomic Library 40

3. MOLECULAR AND BIOLOGICAL ANALYSIS OF BM2 AND BM2L 42

BM2L is a Variant of BM2 42
    Introduction 42
    Results 43
Growth Characteristics in Culture Reflect Differences *In Vivo* 49
    Introduction 49
    Difference in Growth Requirements 50
    Efficiency of Colony Formation 53
    Adhesion and Differentiation Patterns of BM2 and BM2L 53

4. IDENTIFICATION OF GENES INVOLVED IN THE PROGRESSION OF LEUKEMIA 62

Somatic Cell Hybrids 62
    Introduction 62
    Results 62
Differential Gene Expression 66
    Introduction 66
    Results 67
Insertional Mutagenesis 73
    Introduction 73
    Results 73
    Future Directions 94

5. DISCUSSION 97

Introduction 97
    Genes Involved in Signal Transduction are Altered in AML 98

Alteration of Differentiation Genes in AML 101
Oncogene Cooperation and Leukemia 103
Conclusions 105

REFERENCE LIST 108

BIOGRAPHICAL SKETCH 114

LIST OF TABLES

Table page

1. Effect of extracellular matrices on BM2 and BM2L cells 59
2. Biological differences between BM2 and BM2L cells 61
3. Hygromycin drug curve 64
4. Colony formation of Hy/i6 fused cells 65
5. Mechanisms of insertion in the BM2-MAV clones 80
6. Genomic library screening 84

LIST OF FIGURES

Figure page

1. The mechanisms of isertional activation of proto-oncogenes 11

2. Avian Myeloblastosis Virus infection of cells of the monocytic lineage 24

3. Schematic illustration of the origin of BM2 and BM2L cell lines 28

4. Molecular analysis of BM2 and BM2L cells 45

5. Subcellular localization of v-Myb protein in BM2 and BM2L cells . . . 48

6. Difference in growth requirements between BM2 and BM2L cells 52

7. Adhesion and differentiation patterns of BM2 and BM2L cells in the presence of a feeder layer 55

8. Adhesion and differentiation patterns of BM2 and BM2l cells in the presence of LPS and PMA 58

9. Differential display patterns of RNA from BM2 and BM2L cells 68

10. Analysis of differentially expressed cDNA bands 69

11. Molecular analysis of two differential display cDNA bands 71

12. Comparison of growth characteristics among BM2, BM2L and 10.1 cells 76

13. Northern analysis of BM2-MAV clones 78

14. Southern analysis of BM2-MAV clones 81

15. Southern analysis of integrated clone 10.1 83

16. Southern analysis of lambda clone 10.11.1 using an LTR probe 86

17. Sequence analysis of the MAV 3' LTR and cellular junction from 10.1 cells 89

18. Southern analysis of lambda clone 10.11.1 using a cellular junction probe 90

19. Diagram of restriction map of lambda clone 10.11.1 92

20. Southern analysis of BM2 and 10.1 using a cellular junction probe . . . 95

21. Southern analysis of BM2-MAV clones using a cellular junction probe 96

ABBREVIATIONS

AEV	Avian erythroblastosis virus
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ALV	Avian leukemia virus
AMV	Avian myeloblastosis virus
ATL	Adult T-cell leukemia
BM	Normal chicken bone marrow cells
CEC	Chicken embryo cells
cMGF	Chicken myeloid growth factor
CML	Chronic myelogenous leukemia
cpm	Counts per minute
CSF	Colony stimulating factor
Depc	Diethyl pyrocarbonate
DLV	Defective leukemia virus
EGF	Epidermal growth factor
ev	Endogenous virus
HTLV	Human T-lymphotropic virus
IL-3	Interleukein-3

LLV	Lymphoid leukosis virus
LPS	Lipopolysaccharide
LTR	Long terminal repeat
MAV	Myeloblastosis associated virus
m.o.i.	Multiplicity of infection
MuLV	Murine leukemia virus
MMTV	Mouse mammary tumor virus
NBC	New born calf serum
NP	Non-producer
PCR	Polymerase chain reaction
PMA	Phorbol myristate acetate
TPB	Tryptose phosphate broth
pfu	Plaque forming unit
RAV	Rous associated virus
RTA	Reverse transcriptase assay

Abstract of Dissertation Presented to the Graduate School
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ACUTE MYELOID LEUKEMIA: UNDERSTANDING THE GENETIC MECHANISMS

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The mechanism(s) of the progression of Acute Myeloid Leukemia (AML) is still incompletely understood but clearly a complex process that involves multiple control pathways in the cell.

The long term goal of this project is to understand the molecular mechanisms of leukemogenesis and to identify genetic changes involved in the steps necessary for AML progression. An avian model for AML was used which includes BM2 and its variant BM2L, two non-producer, avian myeloblastosis virus (AMV)-transformed cell lines. Injection of BM2 cells into chicken embryos does not induce leukemia, while BM2L cells produce acute myeloblastic leukemia.

BM2L cells spontaneously arose *in vivo* from BM2 cells. At the molecular level, no differences between BM2 and BM2L cells in steady state and protein levels of v-*myb* were observed. *In vitro* assays reflecting the *in vivo* phenotypes of the cells were developed. By measuring the differentiation potential of the cells, only BM2 cells could be induced to differentiate, whereas BM2L cells continued to proliferate. It is possible that BM2L cells cannot be controlled by the bone marrow microenvironment, thus exiting the bone marrow and causing leukemia. In contrast, BM2 cells differentiate in the bone marrow, which could be the reason why these cells are not leukemogenic. Requirements for exogenous growth factors were also measured in culture. BM2L cells form colonies with little requirement for exogenous growth factors, whereas BM2 cells require high levels of serum growth factors for colony formation.

Insertional mutagenesis of BM2 cells by myeloblastosis associated virus (MAV) recapitulates the ability of BM2L cells to form colonies in low serum. After infection, clones with BM2L phenotype were selected by colony formation in 1% serum. Southern analysis of selected clones revealed different patterns of retroviral integrations. One particular clone, 10.1, expressed a novel U5-positive, *env*-negative transcript, which provided an excellent opportunity for identification of the affected cellular gene. Viral cellular junction sequences were isolated from a genomic library generated from 10.1 DNA. Identification of coding sequences in genomic DNA will be necessary to isolate the gene inducing growth factor independence in the 10.1 clone. The gene involved in the establishment of growth factor independence and hence the progression of myeloid leukemia may normally play a role in the program of growth and differentiation of cells from the monocyte/macrophage lineage in hematopoiesis.

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Introduction

The long term goal of my dissertation research was to understand the molecular mechanisms of leukemogenesis, and to identify genes that play a role in the onset and progression of acute myeloid leukemia (AML). The avian model for AML provides an *in vitro* and *in vivo* system to study growth, differentiation, and the analysis of the molecular events that occur in the progression of leukemia. In order to understand what mechanisms are involved in the progression of AML it is important to review what is known about normal hematopoiesis and leukemogenesis.

Normal hematopoiesis is a well-controlled process that leads to the formation of mature blood cells circulating in the peripheral blood or localized in tissues. Proliferation and differentiation of cells from the hematopoietic system is initiated in the bone marrow microenvironment (stroma). The processes are regulated *via* cell-cell interactions, and by the production of specific regulatory molecules such as growth factors (Smith, 1990). Although these mechanisms are extremely complicated, they have been programmed in such a way as to maintain proliferation and differentiation of the cells in a balanced state. In other words, the loss of cells from the stem cell compartment by differentiation into committed progenitor cells must be balanced by replenishment *via* self-renewal of stem cells. If too many cells undergo differentiation, the stem cell reserve will rapidly become exhausted; if too many stem cells undergo self-renewal rather than differentiation, the production of mature cells will drastically fall (Gough and Williams, 1989).

Cells of the monocyte/macrophage lineage are essential in both cellular and humoral immunity, and play an important role in the regulation of human hematopoiesis (Nelson, 1990). During the process of myeloid differentiation, stem cells become committed to myeloid precursor cells and eventually differentiate into functional, morphologically distinct myeloid cells. Little is known about the regulation and maturation of normal myeloid populations and what genes coordinate this process (Lubbert *et al.*, 1991). Contrary to cells of the lymphoid lineage, the traditional classification of myeloid cells has relied almost entirely on morphologic and cytochemical characteristics (Devine and Larson, 1994). The lack of known specific surface markers and genes expressed at the different maturation stages has made their analysis and understanding more complicated (Metcalf, 1989). However, recent advances in immunology, molecular biology and cytogenetics have enhanced the identification of surface markers that distinguish normal hematopoietic cells from their leukemic counterparts. Such technologies have advanced the knowledge of monocyte differentiation and the cellular origins of myeloid leukemias. In addition, molecular biology has made possible the analysis of regulation of expression and characterization of myeloid specific genes such as proteins important for host defense (myeloperoxidase), cytokines (mCSF, interleukins), receptors and adhesion molecules (Lubbert *et al.*, 1991).

Acute Myeloid Leukemia

Introduction

Cancer is the second leading cause of death in the United States (23.7%). Leukemia is a heterogeneous group of diseases that involves hematopoietic cells of the monocytic and lymphocytic lineages with a frequency of approximately 6% among all cancers.

Leukemic cells are characterized by the uncoupling of proliferation and differentiation so that immature progenitor hematopoietic cells accumulate, retain their proliferative capacity, and fail to differentiate into mature blood cells (Gough and Williams, 1989; Sawyers *et al.*, 1991). Some hematologic disorders are not strictly leukemias because they display only part of the full leukemic phenotype, *i.e.* either the uncontrolled proliferation (myeloproliferative syndromes) or the differentiation block (myelodysplasia), yet both can progress to acute leukemia. These disorders have provided the basis for the identification of relevant oncogenic events that occur in a stepwise fashion during the progression of the leukemogenic phenotype (Sawyers *et al.*, 1991).

Human leukemias can be classified as acute and chronic. The acute leukemias can be of lymphocytic or monocytic origin, and are characterized by aberrant differentiation and proliferation of malignantly transformed hematopoietic stem cells.

These cells accumulate within the bone marrow and lead to suppression of the growth and differentiation of normal blood cells. Symptoms result from varying degrees of anemia, neutropenia, and thrombocytopenia, or from infiltration into tissues. The traditional classification of the acute leukemias has relied mainly on morphologic and cytochemical characteristics. However, recent advances in immunology, molecular biology, and cytogenetics have significantly enhanced our understanding of the biologic markers that distinguish normal hematopoietic cells from their leukemic counterparts (Devine and Larson, 1994).

The chronic leukemias include a broad spectrum of disorders. Their clinical manifestations and the approaches to treatment are likewise diverse.

Acute myeloid leukemia (AML) is a bone marrow-based malignancy characterized by maturation arrest of cells at an early stage in myeloid differentiation. The incidence of AML includes approximately 20% of childhood leukemias and 80% of adult leukemias. A diagnosis of AML is established when 30% or more of all the nucleated marrow cells are blast cells. Eight variants of AML have been defined, including three types with mainly granulocytic differentiation (M1, M2, and M3), two with at least 20% monocytic precursors (M4, M5), one with a high proportion of erythroblasts (M6), and a more recently recognized and rarely occurring variant with a predominance of megakaryoblasts (M7).

Molecular Biology of AML

The sequential nature of carcinogenesis has been observed from analysis of samples obtained during tumor progression. For example, studies of events occurring in human colon carcinoma revealed that tumor cells are generally clonal at every stage, consistent with the idea that progression is due to rare genetic events in individual cells of one stage leading to a more advanced stage (Hunter, 1991). The genetic events do not need to occur in a listed order, rather it appears that it is the total accumulation which is important. The number of detectable events increases in parallel with the stage of the disease (Hunter, 1991). The accumulation of multiple genetic lesions is found in other types of human tumors including leukemia. The analysis from animal model systems revealed that the mutational events do not have to occur in a particular order. For example, *ras* mutation in the mouse mammary tumor models is an early event, while in other systems *ras* mutations appear to be a late event (Hunter, 1991). It is the summation of these events that is essential in tumorigenesis including leukemia, supporting the results observed in human colon carcinoma.

A number of lines of evidence support a model of leukemogenesis as a multistep process involving accumulation of multiple genetic alterations. First, clinical manifestations of human leukemias can involve a preleukemic or chronic phase, followed by progression to an acute phase. During the chronic stage, there is an expansion of a precursor cell compartment that retains differentiation capacity, whereas in the acute stage undifferentiated or partially differentiated blast cells circulate in the periphery (Cannistra, 1990; Baer and Bloomfield, 1991). Second, overexpression of growth factors can produce myeloproliferation but not leukemic disorders in animal models, indicating that stimulation of autocrine growth factor production or circumvention of the growth factor requirement may not be sufficient for leukemogenesis (Metcalf, 1989). Third, in human leukemias, cooperativity or synergism among genes is involved in generating the full leukemic phenotype. Fusion of two genes, either *bcr* (breakpoint cluster region) or RAR (retinoic acid receptor) and a new locus *myl* (for myelocytic leukemia), occurs in some myeloid and lymphoblastic leukemias (Cannistra, 1990; Baer and Bloomfield, 1991). Mutations in additional genes, for example p53, N-*ras*, and c-*fms*, have also been found in some human leukemias (Butturini and Gale, 1990).

One way to initiate leukemias is the induction of a dominant mutation in a normal cellular gene termed cellular or proto-oncogene (c-*onc*). The promotion stage is complex, and represents a composite of events involving multiple genetic alterations. These mutations can confer selective advantage to the cells by changing the expression of specific genes, which can induce proliferation of the initiated cell and its clonal descendants, and accumulation of additional genetic alterations (Drinkwater, 1990).

Although the mechanisms of the progression of AMLs are still incompletely understood, clonal abnormalities can be detected in most cases. Advances in cytogenetics have increased our understanding of the association of certain nonrandom chromosomal abnormalities with specific morphologic subtypes of acute leukemia, providing evidence that genetic changes play a major role in the transformation of a normal cell to a neoplastic one. Several oncogenes have been implicated (*i.e. ras, myc, myb*), which clearly shows a complex process that involves multiple control pathways in the cell (Wiernick *et al.*, 1991). These abnormalities include gains or losses of whole chromosomes or the long (q) or short (p) arms of chromosomes, as well as a variety of structural rearrangements (translocations, inversions, or insertions). The most frequent molecular abnormality in AML, occurring in about 25% of cases, is a mutation in the first exon of the N-*ras* gene. The expression of the p53 gene is altered in about half of cases and the RB1 gene is involved in the monocytic variants of AML in about 25% of cases (Cline, 1994).

In order to further understand the possible roles oncogenes may have in the pathogenesis of leukemias, several topics related to the biology of oncogenes will be discussed. These topics include mechanisms of oncogene activation and types of oncogenes; oncogene-induced transformation; oncogenes as a cause of leukemia; and oncogenes and human leukemias.

Oncogenes and Leukemia

Oncogene Activation

Cellular oncogenes can be activated by several complex mechanisms, including retroviral transduction, insertional mutagenesis, translocation, point mutation, deletion and amplification (Butturini and Gale, 1990). Although these mechanisms are often complex and share common features, more than one change may be required for full oncogene activation.

The mechanisms by which oncogenic retroviruses cause tumors or leukemias will be discussed below. These mechanisms include retroviral transduction, insertional mutagenesis, and transactivation.

Retroviral transduction. Retroviruses can incorporate cellular sequences into their genomes. Transduction of cellular oncogenes requires the recombination of retroviral sequences with both 5' and 3' sites in or near the oncogene and deletion of all introns between the transduced exons. This recombination results in a viral genome that contains a new open reading frame encoding a cellular protein. Since all introns are deleted, the transduced cellular sequences resemble cDNAs. Additionally, viral oncogenes (v-*onc*) (cellular sequences incorporated into the virus genome) often differ from their normal homologs because of point mutations, deletions, truncations, insertions, or duplications. These differences could be explained by events that occurred before, during, or after transduction. Structural changes, such as point mutations (v-*ras*), can lead to novel gene products with altered biochemical activity. Deletions or truncations (v-*erbB*, v-*myc*) can cause a loss of a regulatory domain within the oncogene, resulting in constitutive expression or overexpression (Varmus, 1984). Because of constraints on the size of the retrovirus genome, incorporation of cellular sequences is accompanied by deletions of viral sequences. The result is that the retrovirus is replication-defective and requires a helper virus for propagation.

Retroviruses with transduced cellular sequences are rapidly oncogenic in animals, such as birds or mice, and morphologically transform cells in culture. Cell transformation in culture involves changes in cell growth that can be assayed by reduced requirements for serum (growth factors), loss of contact inhibition, and anchorage independence.

Insertional mutagenesis. Retroviruses exert mutagenic effects by integration into the host genome, a natural step in the viral replication cycle. Retroviral insertions commonly result in the activation of host genes or proto-oncogenes that trigger tumorigenesis. The capability of retroviruses to activate gene expression is controlled by the unique properties of *cis*-acting transcriptional regulatory sequences in the LTRs. These sequences, present at both ends of the provirus, contain strong transcriptional promoter/enhancer elements, as well as translational initiation signals and other regulatory elements. Experiments addressing the specificity of provirus integration have shown that insertion occurs preferentially near active genes. The apparent specificity of provirus integration in tumors is actually due to the clonal selection of cells carrying these integration events (Tsichlis and Lazo, 1991). The great majority of insertion mutations will be exclusively heterozygous, even though insertion into tumor suppressor genes will contribute to oncogenesis only if they induce dominant negative mutations or if one allele is already mutated in the cell. Early studies of insertional activation by retroviruses have shown several features that are significant (Kung *et al.*, 1991). First, different viruses can induce similar tumors by interacting with the same oncogene. Second, a single virus can induce different kinds of neoplasia by interacting with different oncogenes. These studies indicated that tissue-specific transformation is mediated by the activation of specific oncogenes and that viral tropism (cell type specificity due to the viral envelope) is not the only factor influencing the wide range of oncogenesis of retroviruses. Third, multiple proto-oncogenes or common insertion sites can be identified in a given type of tumor.

Insertional mutagenesis generally involves altered transcription, although altered processing or stability of transcripts may occasionally have a role (Tsichlis and Lazo, 1991). Generally, four ways of interaction between proviruses and genes have been identified (van Lohuizen and Berns, 1990; Kung *et al.*, 1991) (Figure 1). The first mechanism is promoter insertion (3' LTR promotion). Tumors manifesting 3' LTR promotion have the provirus inserted in the same transcriptional direction as the gene and at sites clustered at the 5' end of the proto-oncogene. The activated oncogene can be detected as a novel U5 positive transcript without any other viral sequences. The most studied example represents ALV proviral insertions occurring predominantly in the first intron of the c-*myc* gene in avian bursal lymphomas (Goodenow and Hayward, 1987; Hayward *et al.*, 1981; Neel *et al.*, 1981). The second mechanism is read-through activation (5' LTR promotion). This mechanism shares some characteristics with 3' LTR promotion, but the oncogene transcripts usually contain viral leader sequences. Transcription extends through the viral genome as well as through cellular sequences downstream from the insertion site. If transcripts escape polyadenylation at the 3' LTR site and use a polyA signal of the downstream proto-oncogene, a fusion transcript is generated containing both viral

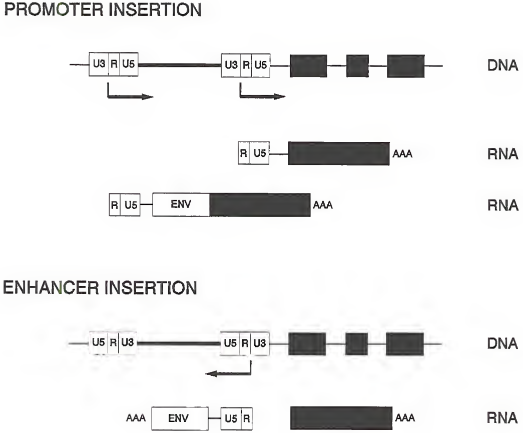


Figure 1. The mechanisms of insertional activation of proto-oncogenes. Cellular regions are represented by black boxes, viral regions are indicated by open boxes. Arrows indicate the direction of transcription. Long terminal repeats (LTRs) are shown as U3, R, and U5. The diagrams with AAA at the end represent the mature transcripts of viral and activated oncogenes.

genes and the flanking oncogene. ALV-integrations in the c-*erb*B/EGF-receptor gene in chicken erythroleukemias, murine leukemia virus (MuLV) integrations in c-*myb* found in mouse lymphosarcomas and MuLV integrations activating the *lck* gene in some murine T-cell lymphomas are well documented examples (Nilsen *et al.*, 1985; Shen-Ong *et al.*, 1986; Adler *et al.*, 1988). Enhancer insertion is the third and most commonly found type of activation because it allows a much larger DNA domain as a target for proviral integrations. The proviruses involved may be located upstream or downstream of activated genes; the provirus does not have an orientation requirement, thus it may be integrated in the opposite transcriptional orientations; and the insertion site may be quite distant from the activated gene. The activated oncogene transcripts are initiated from their natural promoter and the transcript sizes usually are identical to the normal counterpart. Typical examples are the activation of c-*myc* by ALV in some bursal lymphomas, the activation of *int* genes by murine mammary tumor virus (MMTV) in murine mammary carcinomas, and the activation of *pim*-1 and c-*myc* in MuLV-induced T-cell lymphomas (Payne *et al.*, 1982; Peters *et al.*, 1983; Cuypers *et al.*, 1984). The removal of 3' regulating sequences is the fourth mechanism of interaction between proviruses and genes, and may also have a profound effect in the transcription of cellular genes. In some proto-oncogenes proviral insertions are preferentially found in the 3' untranslated region and in the same transcriptional orientation such that the transcript is cleaved at the polyA site of the 5' LTR. The removal of these motifs can confer instability to mRNAs, for example in integrations in the proto-oncogene *pim-1* by MuLV in murine T-cell lymphomas (Domen *et al.*, 1987).

Transactivation. The expression of cellular genes can also be accomplished by virus-encoded proteins. Human T-cell leukemia viruses, type I (HTLV-I) and type II (HTLV-II), are retroviruses associated with adult T-cell leukemia (ATL). Infection by the virus is necessary, but not sufficient, for leukemogenesis. ATL develops in some infected individuals after a latency of many years, suggesting that factors in addition to virus infection contribute to leukemia. The characteristic lymphocyte transforming ability of these viruses is independent of typical oncogenes. Oncogenesis by HTLV-I and related viruses is due in part to the *tax* ($p40^{tax}$) protein, which can transactivate a number of cellular genes, generally playing a role in proliferation (Lazo and Tsichlis, 1990).

Most data suggest that proto-oncogene-encoded proteins regulate normal cell growth and development. Some proto-oncogene products are growth factors or function as mitogens. Other proto-oncogenes encode growth factor receptors; their gene products are transmembrane proteins with both extra- and intracellular domains. A third class of oncogene products are signal transducers; their location is cytoplasmic and they appear to regulate levels of intracellular second messengers. A fourth class of oncogenes encodes nuclear proteins. These proteins are thought to regulate cellular gene expression *via* diverse mechanisms. Additional classes of genes are the suppressor oncogenes and genes that regulate cell death. Tumor suppressor genes differ from the other cases of oncogenes in that it is their absence rather than abnormal expression that is correlated with tumorigenicity (Butturini and Gale, 1990).

Oncogene Induced Transformation

The exact mechanisms whereby proto-oncogene activation results in cellular transformation are unknown. Since oncogenes likely regulate normal cell growth and differentiation, it is not surprising that abnormal proto-oncogene expression might result in unregulated growth. It can be proposed that the disruption of cellular mechanisms such as increased protein kinase or phospholipase activity might alter the balance between cell proliferation and differentiation. Oncogene alterations could also transform cells *via* autocrine stimulation or by constitutive activation of a growth factor receptor pathway. In these models, proliferation might become independent of a normal requirement for an exogenous growth factor. Other oncogenes may regulate DNA replication, cell division or cell death. Independent of the mechanism(s) by which oncogenes induce cell transformation, evolution of a fully malignant phenotype is clearly a multistep process involving multiple alterations within a single oncogene or in several oncogenes. These altered oncogenes may act synergistically on a single metabolic pathway or may dysregulate diverse biochemical pathways.

Oncogenes as a Cause of Leukemia

In order to determine whether oncogenes cause leukemia in humans, it is necessary to show that specific oncogene abnormalities are consistently associated with specific leukemias. Nevertheless, there is variable frequency of oncogene abnormalities in human leukemias. Burkitt leukemia/lymphoma, chronic myelogenous leukemia (CML), Philadelphia (Ph)-chromosome-positive acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), and some cases of B and T-cell ALL and AML show the most consistent oncogene abnormalities. A possible explanation may be that the abnormality is required to initiate but not to maintain the transformed state. Another approach to establishing an etiologic role of oncogenes in human leukemias is to show that these genes transform hematopoietic cells *in vitro* or induce leukemia in experimental animals.

The mechanisms by which oncogenes might cause leukemia are likely to be linked to their proposed role in normal growth regulation. Under these circumstances, it is necessary to consider growth-regulated genes that are expressed in a cell-cycle-dependent manner in normal cells consistent with induction by growth factors. Recent data indicate that several oncogenes, such as *myc, ras, fes, myb, p53* and *fgr*, are growth-regulated and have the ability to transform hematopoietic cells *in vitro*. Several of these oncogenes are aberrantly expressed in leukemias. Whether this is a cause or effect of the changes in cell growth and in the cell cycle is unknown, but both are more than likely. It has also been suggested that multiple oncogene abnormalities can follow a "cascade" model. This model would explain how the same genes can, under different circumstances, transform cells independently without the need to "activate" other oncogenes. Disruption of the balance between dividing, non-dividing and terminally differentiated cells by an uncoupling of proliferation and differentiation is likely important in the link between oncogene abnormalities and leukemogenesis.

Considerable data suggest a role for oncogenes in the pathogenesis of leukemia in experimental models. Infection of animals with retroviruses with and without viral oncogenes induces a wide range of leukemias. Other indirect evidence of oncogene involvement in leukemia comes from experiments with animal or human cells grown *in vitro*. In these models, it is possible to induce growth factor independence *in vitro* or a transformed phenotype *in vitro* or tumors *in vivo* by introduction or manipulation of cellular oncogenes (Tsichlis and Lazo, 1991).

Oncogenes and Human Leukemias

A variety of oncogene abnormalities can occur in human leukemias. These can be classified as qualitative and quantitative, and can result in biochemical or functional alterations in oncogene products. Qualitative alterations in oncogene expression are derived from mutations or deletions in the coding sequences or by formation of a fusion gene due to chromosomal rearrangements. Quantitative abnormalities of oncogenes sometimes result from gene amplification or from numerical chromosome abnormalities, and in general derive from altered gene expression. For example, insertional mutagenesis can also result in quantitative abnormalities in gene expression (Butturini and Gale, 1990). However, these different pathways of oncogene activation are not mutually exclusive and sometimes single genetic events may have various consequences. For example, translocation of MYC in different tumors can result in increased expression, inappropriate pattern of expression, or changes in mRNA or protein stability (Payne *et al.*, 1982).

Animal Models for AML

Introduction

AML is the least studied and understood of the acute leukemias because no AML-specific surface antigens have been identified. Instead, AML cells often express antigens simultaneously on the cell surface that are not normally expressed by the normal counterparts. In addition, the study of human AMLs has also been impaired because of the difficulty in establishing human cell lines in culture, despite the impressive proliferation rates in patients (Baer and Bloomfield, 1991). The availability of animal models for acute myeloid leukemia circumvents this problem.

The avian hematopoietic system has provided a unique and interesting model to study mechanisms of the regulation of cell proliferation and differentiation in normal versus tumor cells. One of the advantages in using avian models is that hematopoiesis in chickens is regulated by similar pathways as those found in other mammalian systems including humans. For instance, avian hematopoietic cells arise by proliferation and differentiation of the progenitor cells. This process begins with multipotential stem cells that can self-renew and undergo progressive differentiation to cells committed to a particular lineage, ultimately yielding mature blood cells (Metcalf and Moore, 1971). Commitment is an irreversible step whereby progenitor cells have lost the potential to generate hematopoietic cells of other lineages. Most proliferative activity in the bone marrow seems to occur in the progenitor cells committed to the production of single or restricted ranges of hematopoietic cell types. Analysis of the stem cells and progenitor cells in different hematopoietic tissues has been useful in clarifying the differentiation pathway as well as in exploring the regulatory mechanisms of hematopoiesis. Hematopoietic cell differentiation can be triggered, for example, in response to infection. Progenitor cells in the bone marrow would rapidly produce a large amount of mature granulocytes and monocytes under the control of a variety of cytokines and colony-stimulating factors (CSFs). In the murine and human system, four kinds of CSFs involved in myelopoiesis have been characterized as IL3, GM-CSF, G-CSF and M-CSF. Conversely, the specific CSFs in the chicken have yet to be identified. Nevertheless, sources of CSFs can be furnished by using a feeder layer of macrophages (Graf *et al.*, 1981), or a conditioned medium from chicken fibroblast cultures (Dodge and Moscovici, 1973; Dodge *et al.*, 1975; Gazzolo *et al.*, 1979). Cells transformed by the avian myeloblastosis virus (AMV) were also shown to be capable of producing CSFs (Silva *et al.*, 1976). Recently a myelomonocytic growth factor was isolated from medium conditioned by a transformed macrophage cell line (Leutz *et al.*, 1984). This factor (c-MGF) promotes the growth of macrophage colonies together with a minor proportion of granulocytes.

In addition, hematopoiesis in chickens can be studied both *in vivo* and *in vitro* by using retroviruses as indicators for specific precursors present within each lineage.

Studies of murine and avian retroviruses have been critical for understanding molecular mechanisms of human oncogenesis, even though most human malignancies arise independent of retroviruses. However, many of the genes involved in the multistep events leading to human malignancies were originally identified because of animal retroviruses. In addition, genetic changes such as point mutations and translocations activate or change human proto-oncogenes, similar to the effects of retroviruses in animal tumorigenesis.

Retroviruses are a heterogeneous group of RNA viruses that have a distinct pattern of replication. These viruses have been isolated from a variety of vertebrates, including, mice, birds, cats, nonhuman primates, and humans. Upon entering the cell, retroviruses synthesize a DNA copy of their genome (provirus), which integrates into the cellular DNA. Following integration, cellular enzymes synthesize virus-specific mRNA transcripts that are translated into proteins. The viral proteins package the viral genomic RNA, and the viral particles exit the cell by budding through the cellular membrane, usually causing no cytotoxic effect on the infected cells (Weiss *et al.*, 1982; Weiss *et al.*, 1985).

Retroviruses can be divided into endogenous and exogenous retroviruses based on their mechanism of transmission. Contrary to the exogenous retroviruses, which are transmitted horizontally, the endogenous retroviruses are integrated into the cellular genome and transmitted vertically, like other cellular genes. At least sixteen independent endogenous viral (ev) loci have been identified in chicken cells. These DNA sequences are closely related to the Rous-associated virus-0 (RAV-0).

All retroviruses share some basic features of their genomic organization. All contain a DNA sequence called a long terminal repeat (LTR) that is repeated at both ends of the proviral DNA. The LTR is composed of three elements, U_3, R and U_5, which correspond to the retroviral promoter. Between the two LTRs, all replication-competent viruses contain at least three genes, *gag* coding for the viral structural genes, *pol* coding for reverse transcriptase, integrase and protease, and *env* coding for the viral envelope glycoproteins (Lazo and Tsichlis, 1990).

Cell lines transformed by avian leukemia viruses have provided important animal models for analyzing the leukemic process. Infection by acute leukemia viruses leads to proliferation and transformation of cells that display distinct phenotypes of hematopoietic lineages (Moscovici and Gazzolo, 1982). For example, the avian myeloblastosis virus (AMV) leads to the transformation of cells from the myeloid lineage specifically inducing acute myeloid leukemia in the chicken.

Avian Leukemia Viruses

The avian leukemia viruses (ALVs) belong to a taxonomic subfamily termed oncoviridae (avian leukosis-sarcoma group of type C RNA tumor viruses) within the family of retroviruses (Fenner, 1976). They contribute to a variety of avian hematopoietic and non-hematopoietic disorders. ALVs can be divided into 2 groups according to the pathological features: (1) the defective leukemia viruses (DLVs) (also known as acute leukemia viruses); and (2) the avian leukosis viruses, also referred to as lymphoid leukosis viruses (LLV) or helper viruses (Hanafusa, 1977).

Avian leukemia viruses are recognized as the etiological agents for causing a large spectrum of diseases including leukemias in chickens. The group of ALVs called defective leukemia viruses are the more widely used in the study of leukemogenesis. These transforming viruses are derivatives of the leukosis viruses, but have replaced replicative viral genes by the transduction of usually one, and in some cases, multiple cellular oncogenes (Chen *et al.*, 1980; Bister and Duesberg, 1980). Therefore, the acute leukemia viruses are defective for replication and can produce infectious progeny only when a leukosis helper virus provides replicative functions in *trans* (Moscovici and Gazzolo, 1982). DLVs induce a rapid disease *in vivo* and also have the ability to transform cells *in vitro*. Tumor induction or leukemia caused by these viruses is due to the direct action of the oncogenes they carry. *In vitro* studies of transformation of hematopoietic cells by these viruses have led to a better understanding of the oncobiology of the DLVs (Bister and Duesberg, 1980). Based on the predominant response of the hematopoietic system of the infected host and the major types of oncogenes which they carry, three subgroups of DLVs and their respective representatives can be distinguished: 1) the MC29 subgroup: myelocytomatosis (v-*myc*); 2) the AEV group: erythroblastosis (v-*erb*) and the AMV group: myeloblastosis (v-*myb*). The AMV group will be discussed in detail.

Insights into the mechanisms by which oncogenes are able to mediate the transformation of hematopoietic cells were provided by several areas of study. First, the characterization and identification of the target cells for neoplastic transformation of DLVs and second, by the study of the phenotype of the cells transformed by these viruses. These studies have been made possible because of the development of assays that determined *in vitro* the transforming capability of these viruses. Examples of some of these *in vitro* assays are: lineage-specific transformation, target cell specificity (Gazzolo *et al.*, 1979), differentiation parameters, colony assays (Gazzolo *et al.*, 1979), and growth factor independence.

In contrast to the DLVs, the lymphoid leukosis viruses (LLVs) do not contain any v-*onc* sequences and they are fully competent for replication. Because of the absence of v-*onc*, the LLVs do not transform cells *in vitro* and most strains induce predominantly lymphoid leukosis *in vivo* only after a long latent period of several months or longer by the activation of proto-oncogenes.

Avian Myeloblastosis Virus

Avian myeloblastosis virus (AMV), which is a DLV containing the v-*myb* oncogene, is the only strain of virus known to induce exclusively an acute monoblastic leukemia in the chicken and to transform hematopoietic cells to the monocytic lineage *in vitro*. AMV is the only retrovirus with the ability to replicate in chicken embryo fibroblasts without inducing their transformation. AMV can be divided into subgroups A and B, depending on the expression of envelope glycoproteins of the helper viruses (Moscovici, 1975). In order to replicate and produce infectious progeny, AMV requires one of its two helper leukosis viruses, the myeloblastosis associated virus 1 or 2 (MAV-1, MAV-2) of subgroups A and B, respectively (Moscovici and Vogt, 1968).

AMV target cells have been characterized by density, velocity sedimentation, adherence, and phagocytic activity. These parameters indicate that cells are recruited from cells within the monocytic lineage, from the stage of the myelomonocytic progenitors committed toward the macrophage lineage (Boettiger and Durban, 1984) to the terminally differentiated macrophages (Moscovici and Gazzolo, 1982). Regardless of the origin of the target cells, the AMV-transformed cells are morphologically identical, and possess the same functional and surface properties (Beug *et al.*, 1979; Gazzolo *et al.*, 1979; Durban and Boettiger, 1981) (Figure 2). The cells are mostly non-adherent and round with a large, eccentric nucleus that is surrounded by a rim of cytoplasm containing small granules. The receptors for the Fc region of immunoglobulins are expressed on the cell surface, whereas C3 receptors, normally expressed in avian macrophages, are not present. Although the AMV-transformed cells engulf latex particles mediated by non-specific receptors, phagocytosis mediated by Fc receptors does not occur (Fc receptors are not functional). Acid phosphatase and adenosine triphosphatase (ATPase) are also found in the cytoplasm and on the membrane of the transformed cells. Moreover, after treatment with phorbol ester (PMA), which has been previously shown to induce myeloid cell differentiation, the AMV-transformed cells become adherent to the surface of the culture flask and eventually will differentiate into macrophages (Pessano *et al.*, 1979; Symonds *et al.*, 1984). There were no obvious alterations in terms of the expression of the v-*myb* proteins in these PMA-differentiated cells. However, the v-*myb* proteins were found to be located in the cytoplasm instead of in the nucleus (Symonds *et al.*, 1984). Differentiation into macrophages was also observed in cells containing a temperature-sensitive allele of v-*myb* when they were

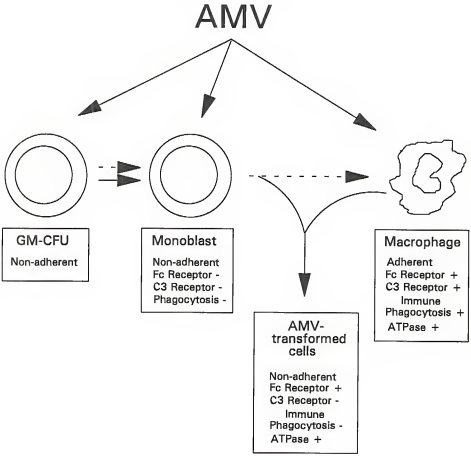


Figure 2. Avian myeloblastosis virus infection of cells of the monocytic lineage. Cells from different stages of the monocytic lineage can serve as the target cells for AMV transformation. The transformed cells are arrested at the stage between monoblast and monocyte *via* either partial differentiation or dedifferentiation. These cells are non-adherent and round-shaped. The ATPases are expressed on their cell surface, whereas C3 receptors are not. Although they have Fc receptors, immune-phagocytosis cannot be mediated by these non-functional receptors. Dotted arrows represent the normal monocyte differentiation pathway. Solid arrows represent the differentiation and dedifferentiation pathways induced by AMV.

shifted to a nonpermissive temperature. These results suggest that Myb protein is directly involved in the regulation of the proliferation/differentiation pathway of myeloid cells (Moscovici and Moscovici, 1983; Schirm *et al.*, 1990).

In conclusion, cells from all stages of the monocytic lineage, from the committed progenitors to the mature macrophages, may serve as the target cells for AMV. Once the cells are transformed, they are blocked at a stage between monoblasts and monocytes (Figure 2).

The lineage-specific transformation of AMV is due to the oncogene v-*myb*, which encodes a transforming protein p45-*myb* located in the nucleus (Klempnauer *et al.*, 1984). The v-*myb* gene is a truncated version of the proto-oncogene c-*myb* lacking coding sequences from both the 5' and 3' ends. The truncated protein product of v-*myb* lacks part of the DNA binding domain as well as a negative regulatory domain. The exact function of v-Myb proteins remains to be clearly established. However, recent results indicate that v-Myb along with c-Myb can bind DNA with sequence specificity and function as a regulator of gene expression by binding directly to *myb* target genes (Boyle *et al.*, 1985; Weston and Bishop, 1989).

Expression of *myb* is found predominantly in immature cells and it is shut off in mature cells and upon terminal differentiation. It appears that *myb* will transform or block differentiation of cells only when it is expressed at particular stages of certain cell types. Therefore, Myb may normally function as an important regulatory protein in cell development. One can hypothesize that Myb functions by transactivating genes that in turn are only expressed in a lineage- and maturation stage-specific manner. Alternatively, Myb may repress genes that are only expressed in terminally differentiating cells (Shen-Ong, 1990).

The BM2/BM2L Model

The majority of transformed cell lines are virus producers, expressing both acute leukemia viruses, which are replication-defective, and replication-competent helper viruses. As a consequence, there is continuous infection and transformation of new cells as the viruses proliferate *in vivo*. Availability of a non-producer AMV transformed cell line could circumvent the complication of virus production and would provide a model to study proliferation, differentiation, and the role exerted by a normal host microenvironment on proliferating transformed cells.

BM2 is a non-producer (NP) AMV-transformed chicken cell line established by Drs. C. and M.G. Moscovici (Moscovici, 1975; Moscovici and Moscovici, 1980). This myeloblastic cell line originated from the bone marrow of a 2 week-old bird injected with the NP cell line 727. Line 727 was obtained by infection of 17-day-old SPAFAS embryonic bone marrow cells with AMV-B at a low multiplicity of infection (m.o.i. of 0.01 to 0.001). The infected cells were cloned by limiting dilution and supernatant fluids were tested for the production of virus. Transformation of BM2 cells is observed *in vitro* by the ability of the cells to grow indefinitely. However, the stage of immortalization can be dissociated from tumorigenesis *in vivo*. When injected into chicken embryos, BM2 cells home to the bone marrow but are unable to produce overt leukemia (Moscovici *et al.*, 1982; Bagnis *et al.*, 1993).

The non-producer cells are indistinguishable from the virus producers in their growth requirements, mode of replication, surface markers, and colony formation (Moscovici and Moscovici, 1980). The non-producer cell lines differ by the lack of infectious virions, tested by both the reverse transcriptase assay (RTA) and conventional biological assays (Moscovici, 1975; Moscovici *et al.*, 1976). Recently, a cell variant was recovered from the bone marrow of one of the birds injected with a late passage of BM2 cells. When reinjected into the chicken embryo, these variant cells caused an acute monoblastic leukemia. Cells retrieved from the bone marrow gave rise to the stable non-producer leukemogenic cell line BM2L (Figure 3). Both cell lines are monoblasts which maintained the differentiation parameters as wild type AMV-transformed cells (Moscovici *et al.*, 1982).

In the BM2/BM2L model, the BM2L cells combine their uncontrolled growth with a defect in cell maturation and function which leads to leukemia. This dissertation project tests the hypothesis that the two cell lines differ in the pathways leading to cell differentiation and maturation. BM2 cells when injected into the chick embryo are fully capable of differentiating, while the BM2L cells do not differentiate leading to leukemia and formation of malignant foci in the liver and spleen (Moscovici *et al.*, 1982). Thus, BM2 and BM2L cell lines are identical except for a defect in a differentiation/maturation pathway, this providing a useful opportunity for the study of differential gene expression in the leukemic process.

In summary, leukemogenesis can be viewed as essentially a two-step process (Sawyers *et al.*, 1991), involving growth of the cell population and a defect in the

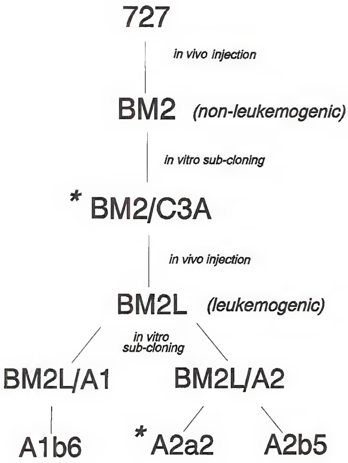


Figure 3. Schematic illustration of the origin of BM2 and BM2L cell lines. BM2 and BM2L cells were recovered and cultured *in vitro* from the bone marrow of injected birds. The asterisk denotes the two representative clones used in all of my experiments, which I will refer to as BM2 and BM2L cells.

differentiation of the cells. These events would involve alterations in the normal patterns of expression of genes involved in regulatory pathways. Several models have been formulated to account for what types of genes may be involved in the leukemogenicity of BM2L cells. First, altered genes could mimic signalling by ligand-activated growth factor receptors and thus cause factor-dependent proliferation (non-autocrine mechanism). Second, genes could induce factor-independent growth either by encoding a growth factor themselves or by inducing the synthesis of a growth factor which is not normally produced by the cell (autocrine mechanism). Third, genes could alter the normal proliferation/differentiation program of the cells, for example by inducing proliferation or preventing differentiation.

This dissertation focuses on the understanding of the regulation of myeloid differentiation to try to identify genes that may play a positive or negative role in the progression of myeloid leukemia. In order to achieve the main goal, my dissertation research has focused on the following specific aims: (1) to determine whether the leukemogenic BM2L cell line is a true variant of BM2, or whether the cells arose from a spontaneous leukemia in the bird; (2) to determine the role of *v-myb* in the leukemogenic progression; (3) to establish an *in vitro* assay that reflects the biological differences between BM2 and BM2L in order to study the genetic mechanisms involved in the progression of AML; (4) to determine whether the leukemogenic phenotype is dominant or recessive; and (5) to identify genes involved in the induction or suppression of AML.

CHAPTER 2

MATERIALS AND METHODS

Southern Analysis

Genomic DNA was extracted by standard methods (Sambrook *et al.*, 1989) and digested with restriction enzymes according to the manufacturer's protocols (Gibco, BRL). Restricted DNA was electrophoresed in 1% agarose gels and transferred to NYTRAN membrane (Schleicher and Schuell) according to the method of Southern (Southern, 1975). Probes were prepared from double-stranded fragments, which were excised with appropriate restriction enzymes from plasmid vectors, labelled with ^{32}P-dATP to a specific activity of at least 3 X 10^9 cpm / μg DNA by the random primed method (Feinberg and Vogelstein, 1984), and separated from unincorporated nucleotides by Sephadex G-50 (Pharmacia) spun column (Sambrook *et al.*, 1989). Filters were prehybridized for 1 hour at 60° in phosphate hybridization buffer (0.5 M $NaPO_4$ pH 7.2, 0.5 M EDTA pH 8.0, 7% SDS, 1% BSA) (Church and Gilbert, 1984) followed by 16 hours at 60°C with 1 X 10^6 cpm of radiolabelled probe per ml of buffer. After washing for one hour at 60°C in 40 mM $NaPO_4$ pH 7.2 with 0.1% SDS, filters were exposed to Kodak XAR 5 film with intensifying screens at -70°C.

Sequencing

Oligonucleotide primers specific for v-*myb* were synthesized on an Applied Biosystems DNA synthesizer in the DNA synthesis core facility at the University of Florida. Sequences of primers used in amplification reactions and their location in AMV flanking the v-*myb gene* (Klempnauer *et al.*, 1982) are as follows:

MG90: 5'- CGGGATCCAAGTTAAACCGGACATCGCCC - 3' (33-53)
MG89: 5'- GAAGATCTCCGCCGTGCATCTTCCTGTAT - 3' (1326-1306)
MG135: 5' - TAATGCCTGCTGCCCTTTCA - 3' (345-364)
MG136: 5' - GAAGGATTGGAAAACAGTGC - 3' (919-900)

Underlined nucleotides are restriction sites (*Bam*HI in MG90 and *Bgl*II in MG89) incorporated into the primers to facilitate cloning of the amplified products.

V-*myb* sequences were amplified from 1 μg of DNA in 100 μl reactions with PCR buffer (1.5 mM magnesium chloride, 50 mM potassium chloride, 20 mM Tris pH 8.4, 0.1% BSA), 1 μM of each primer, 10 mM dNTPs, and 2.5 units Amplitaq (Perkin Elmer) overlaid with 20 μl mineral oil (Saiki *et al.*, 1985; Grimes *et al.*, 1992). Amplification reactions were carried out using an automated 48-well thermal cycler (Perkin Elmer Cetus) programmed for one cycle of denaturing (94°C for 10 min), 35 cycles of amplification (denaturation for 1 min at 94°C, 1 min primer annealing at 60°C and 2 min elongation at 72°C) followed by one cycle of polymerization (72°C for 10 min). Amplified DNA fragments were purified from agarose gels and directionally subcloned into pGEM-3Z (Promega) for double-stranded sequencing with Sequenase Version 2.0 (United States Biochemical) according to the manufacturer's protocols.

RNA Extraction and Northern Blots

RNA was extracted from cells in culture according to standard methods (Sambrook *et al.*, 1989). Cell pellets were processed in a Virtis homogenizer in guanidine thiocyanate buffer (4 M guanidine thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% Sarkosyl, 0.1 M ß-mercaptoethanol). The homogenate was layered onto CsCl (5.7 M CsCl, 0.1 M NaOAc pH 5.0, 100 mM EDTA). RNA was pelleted by ultracentrifugation at 330,000 x g for 16 hours in a Beckman SW40 rotor and resuspended in water for total RNA or in ETS (5 mM EDTA, 10 mM Tris, 0.05% SDS) for mRNA. Poly(A) RNA was selected by passing the sample over an oligo(dT)-cellulose column. After ethanol precipitation, RNA was resuspended in water and quantitated by absorbance at 260 nm on a Beckman DU-64 spectrophotometer. For Northern analysis, 5μg of poly(A) RNA samples were denatured at 50°C for 30 min in glyoxal buffer (5.7% glyoxal, 50% dimethyl sulfoxide, 10 mM disodium dihydrogen phosphate) and electrophoresed in a 1% agarose gel in 10 mM sodium dihydrogen phosphate buffer. Gels were blotted by capillary transfer onto NYTRAN membrane (Schleicher and Schuell), baked at 80°C for 2 hours, prehybridized and hybridized as described for the Southern analysis.

Plasmids and Probes

A *myb*-specific probe was obtained by isolating and subcloning a 220-bp *Sma*I-*Sal*I cDNA fragment from pVM2 (Klempnauer *et al.*, 1982; Klempnauer *et al.*, 1983). Viral probes included: U5L LTR, a 315 bp *Eco*RI-*Sac*I subclone of plasmid p5'NCR; LDR, a 175 bp *Eco*RI-*Bst*EII subclone of plasmid pU5L (Goodenow and Hayward, 1987); a 1.2 kb *Bam*HI-*Kpn*I subclone of pAMVpol-*myb* (Klempnauer *et al.*, 1982; Klempnauer *et al.*, 1983); and a 564 bp *Rsa*I subclone of plasmid pMAVenv (unpublished). The plasmids pVM2 and pAMVpol-*myb* were kindly provided by Dr. Sabine Schirm. The plasmid tgCMV/HyTK used in the somatic cell fusions was kindly provided by Targeted Genetics, Seattle, Washington.

Western Analysis

Extracts of nuclear proteins were prepared by resuspending cell pellets in 5 ml of hypotonic buffer (25 mM Tris pH 7.4-8.0, 5 mM KCl, 1 mM $MgCl_2$) and incubating on ice for 15 min. After spinning at 1000 x g for 5 min, the pellet was resuspended in 2 to 3 ml of lysis buffer (50 mM Tris pH 7.5-8.0, 5 mM EDTA, 10 mM NaCl, 0.5% NP40), incubated 2-5 min on ice, and centrifuged at 1000 x g for 5 min. Nuclei were lysed in Läemmli-buffer (Läemmli, 1970). The nuclear extract equivalent to 5 x 10^6 cells was resolved by electrophoresis on 7.5% SDS-PAGE gels (Läemmli, 1970) and then transferred to nitrocellulose filters. Filters were incubated with 5% BSA in Tris-buffered saline (TBS) (25 mM Tris) overnight and probed with anti-Myb monoclonal antibody (1:500) (Evan *et al.*, 1984), obtained from Dr. Sabine

Schirm and with goat anti-mouse IgG antibody-alkaline phosphatase (calf intestine) conjugate (Boehringer) according to standard protocols (Sambrook *et al.*, 1989).

Immunofluorescence

Cells growing in suspension were pelleted onto glass slides by cytospin. Cells attached to the coverslips were washed with phosphate-buffered saline (PBS) and fixed with 100% methanol for 2 min at room temperature. After brief washes with PBS, cells were then incubated with a blocking solution of 1% BSA for 30 min at 37°C. Cells were washed extensively in PBS before probing for 30 min at 37°C with anti-Myb polyclonal antibody (1:100) obtained from Dr. Sabine Schirm, or normal rabbit serum (1:100). Cells were extensively washed again and probed with a dilution of 1:1000 of fluorescein-isothiocyanate (FITC)- conjugated goat anti-rabbit IgG antibody. Slides were dried and mounted with a solution of 50% glycerol.

Cell Lines and Growth Media

Cells were grown in Dulbecco's modified eagle medium (DMEM) standard or depleted (Gibco). Two types of DMEM standard medium were used, both containing 10% tryptose phosphate broth (TPB) and 0.1% gentamicin (10 mg/ml): standard high (5% chicken serum, 5% calf serum) and standard low (0.5% chicken serum, 0.5% calf serum). DMEM-depleted medium contained folic acid (160 μg/100ml), 1% vitamins (100x) and 0.1% gentamicin (10 mg/ml). Two types of medium were used: depleted high (10% newborn calf serum) (NBC) and depleted low (1% NBC serum).

In this dissertation cells referred as BM2 correspond to the clone BM2/C3A. These cells were grown in DMEM standard high medium. BM2L cells refer to the clone BM2L/A2a2, which were grown in DMEM standard high medium or DMEM depleted high medium. All cell cultures were incubated at 37°C in a humid atmosphere with 5% CO_2. For growth curves, cells were seeded in triplicate 60-mm dishes at 2 X 10^6 cells per ml of growth medium. Viable cells were counted by trypan blue dye exclusion every 24 hours for a period of four days.

The methylcellulose colony technique was used to isolate clones *in vitro* and to assay the efficiency of colony formation of the cell lines. 5 X 10^4 cells were dispersed in 2 ml of 4% methylcellulose containing either DMEM standard high or low medium. Assays were done in triplicate and the average number of colonies is reported.

Normal bone marrow cells were obtained by flushing out of tibias with standard medium (Jurdic *et al.*, 1982), and filtering through a nylon mesh. Chicken embryo cells were obtained from 10-day old embryos according to Vogt (1969).

In Vivo Experiments

Both the non-leukemogenic and leukemogenic cells were injected at a concentration of 1 X 10^6 cells in 0.1 ml of medium into 11-day embryos (C/E SPAFAS Inc., Norwich, Conn.) *via* the chorioallantoic vein (Moscovici *et al.*, 1982). Blood smears were obtained daily from all hatched chickens.

Differentiation Assays

BM2 and BM2L cells seeded at 1 X 10^5 per ml of DMEM standard high medium in 60-mm dishes were cocultivated with a primary culture of chicken embryo cells (CEC) or freshly seeded bone marrow cells (BM) at a concentration of 2 X 10^6 cells per dish for a period of five days.

Partial differentiation was established by seeding 2 X 10^6 BM2 cells per 60-mm dish in standard medium containing 10 μg of lipopolysaccharide (LPS) (Sigma). The majority of cells (aprox. 80%) adhered to the plate with extended processes by day 2 after seeding. The remainder of the cells were blasts and macrophages. Terminal differentiation to a macrophage-like phenotype was obtained by seeding the same number of cells in standard medium containing LPS and 250 ng/ml of phorbol ester (PMA) (Sigma).

Adhesion and differentiation was screened by comparing (1) the morphology of the cells to the controls (CEC and BM cells alone or cells without LPS or PMA) and (2) counting the number of residual cells in the supernatant fluids as well as the total number of attached cells after trypsinization of the cultures.

The same experiments were done using individual components of the extracellular matrix, including fibronectin, laminin, collagen type I rat tail, and matrigel (Biocoat, CRI).

Electroporation

Suspension cells were grown overnight in RPMI 1640 medium (Gibco) with 20% total serum. On the next day cells were recovered on a ficoll gradient and washed extensively with PBS. Cells were then seeded at a concentration of 2 X 10^6 per ml in RPMI 1640 with 20% serum (10% chicken, 10% calf serum) and incubated for two hours at 37°C. After the incubation period, cells were pelleted and resuspended in 250 μl of complete media. The vector DNA (20 μg/5 μl) and cell suspension were added to the electroporation cuvette (Biorad) and chilled on ice for 2 min. Electroporation was done at 0.3kV after which the cells were placed on ice for at least 10 minutes. Transfected cells were then seeded in 10 mls of RPMI 1640 with 20% serum for 48 hours. After 48 hours drug selection was started, and after 4 days in culture the media was changed to DMEM standard. Resistant cells were cloned in methylcellulose media after 3 weeks in culture.

Somatic Cell Fusions

Cell fusions were carried out as described previously (Pontecorvo, 1975; Davidson and Gerald, 1976). Briefly, 5 X 10^6 BM2Hy and 5 X 10^6 BM2Li6 cells were washed in PBS and mixed together. One ml of 50% PEG (37°C) was added to the pellet over a period of 1 min, allowed to stand 30 sec, and then diluted by adding 9 ml of serum-free media. Cells were washed several times with serum-free media and resuspended in 4 ml of complete media. Cells were then incubated at 37°C for 48 hours without drug selection. A colony assay with double drug selection (1 mg/ml

G418 (sigma; 250 μg/ml hygromycin B (Boehringer Manheim)) was done after 48 hrs using 5 X 10^5 and 2 X 10^5 cells per dish. A drug curve using different concentrations of hygromycin B and G418 was done to determine the amount to be used for this assay (Table 3). The concentration of G418 for the BM2Li6 cells was established by Dr. Bagnis (Bagnis *et al.*, 1993). As controls, colony assays were done with BM2Hy cells with G418 selection and BM2Li6 cells with hygromycin selection.

Differential Display

The removal of chromosomal DNA contaminating the RNA was performed by incubating the total RNA with 10 units of DNase I (Boehringer) in 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM magnesium chloride. After extraction with phenol/chloroform, the supernatant was ethanol precipitated in the presence of 0.3 M sodium acetate and the RNA was redissolved indiethyl pyrocarbonate (DEPC)-treated water. Differential display was performed by using a RNAmap kit (GenHunter Corporation) as described previously (Liang and Pardee, 1992; Liang *et al.*, 1993) or performed by the Differential Display Core Laboratory, ICBR at the University of Florida. Briefly, 0.1 μg of total RNA was used for reverse transcription in a 20μl reaction volume. One tenth of the RT reaction was used in the polymerase chain reaction. The amplified cDNAs were then separated on a 6% DNA sequencing gel. The DNA sequencing gel was blotted onto a piece of Whatman 3MM paper and dried without methanol/acetic acid fixing. The autoradiogram and dried gel were oriented with needle punches. After developing the film, cDNA bands of interest were located by cutting through the film. The gel slice along with the 3MM paper was incubated in 100μl distilled water for 10 min. After rehydration of the polyacrylamide gel, the cDNA was diffused out by boiling the gel slice for 15 min in a tightly capped microfuge tube. cDNA was recovered by ethanol precipitation in the presence of 0.3M sodium acetate plus 5 μl of 10 mg/ml glycogen as a carrier and redissolved in 10 μl of water. The eluted cDNA (4 μl) was reamplified in a 40 μl reaction volume using the same primer set and PCR conditions used in the display reaction. Reamplified cDNA probes were cloned into the PCRII vector using the TA cloning system from Invitrogen, and then used to hybridize Southern and Northern blots.

Retroviral Infections

Infections were performed according to the protocol described by Moscovici *et al.* (1983). Briefly, BM2 cells were infected with MAV-2 virus at high m.o.i.. The virus adsorption was carried out 30 min at 4°C. Cells were cultured in standard medium for 48 hours and checked for integrations by PCR using primers flanking the *env* gene (MG89, MG90). Cells with the MAV-2 virus integrated in their genome were cloned in methylcellulose containing DMEM standard low medium (1% serum). The colonies were picked after six days in culture and 9 independent clones were established (M 3.1, M 9.1, M 9.5, M 10.1, M 10.3, M 10.6, M 11.1, M 11.3, and M 11.4).

Genomic Library

After fractionating partial *Eco* RI-digested genomic DNA (100 μg) of the clone M 10.1 on a 10-40% sucrose density gradient, a genomic library was constructed from DNA isolated from the clone BM2-10.1 that harbored one proviral insertion. The 18-23 kb fractions were ligated to *Eco*RI-digested EMBL4 arms (Pharmacia), and, after packaging, the library was amplified on *E. coli* strain XL1-Blue MRA (P2) (Pharmacia) with a representation of at least 10 genome equivalents (3.0×10^{10} nucleotides, where the chicken haploid genome contains 1×10^9 nucleotides). The library (6×10^5 independent plaques) was plated on *E. coli* strain XL1-Blue MRA (Pharmacia) and hybridized to a viral U5L LTR probe (Goodenow and Hayward, 1987). Filters were pre-washed for 30 min at 60°C in 50mM Tris pH 8.0, 1M sodium chloride, 0.1% sodium dodecyl sulphate (SDS), and 1mM EDTA. Prehybridization and hybridization was done at 60°C in 0.1% bovine serum albumin, 0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% SDS, 6X SSC, and 50 μg/ml denatured salmon sperm DNA. After 16-18 hours of hybridization, filters were washed for 10 min at 60°C in 2X SSC and 0.1% SDS and for 3 hours in 1X SSC and 0.1% SDS. Phage DNA from positive clones was isolated as described previously (Sambrook *et al.*, 1989) except that plate lysates were done using Seakem LE (FMC) as top agarose and phage was precipitated using Lambdasorb (Promega). Phage DNA was further analyzed with a panel of restriction enzymes. Lambda phage DNA was prepared using a QIAGEN Lambda kit. Junction fragments were subcloned into pGEM-3Z (Promega) for double-stranded sequencing, restriction analysis, and construction of double stranded probes.

CHAPTER 3

MOLECULAR AND BIOLOGICAL ANALYSIS OF BM2 AND BM2L

BM2L is a Variant of BM2

Introduction

In order to define whether BM2 and BM2L cells could provide a model for the identification of genes that play a positive or negative role in regulation of the leukemic process, the study of molecular and biological properties of these cells was necessary.

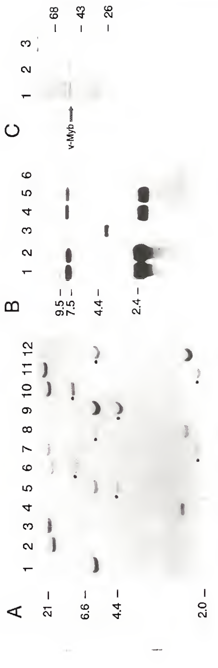
My first goal was to determine whether the leukemogenic BM2L cell line was a true variant of BM2, or whether it arose from a spontaneous leukemia in the bird. Leukemogenic variants of BM2 cells should contain v-*myb* sequences integrated in their genome, whereas spontaneous leukemia cells would not. Therefore, I took advantage of the v-*myb* sequences in BM2 cells as a genetic marker to analyze the DNA of BM2L cells. Consequently, if BM2L is a variant of BM2 cells, the role of v-*myb* in the leukemogenic progression would be analyzed.

Results

Molecular differences between BM2 and BM2L cells were evaluated by Southern analysis using a *myb* cDNA probe, which is able to detect both c-*myb* and v-*myb* sequences. The first result was that BM2L cells contained the v-*myb* gene integrated into their genome (Figure 4). The presence of the v-*myb* lineage marker indicated that BM2L cells were true variants that arose spontaneously from the BM2 cell line. Second, rearrangements of v-*myb* or c-*myb* were not detected in either cell line. Genomic DNA from BM2 and BM2L cells was digested with *Hin*dIII or *Kpn*I for internal c-*myb* or v-*myb* fragments, and with *Bam*HI or *Eco*RI for regions flanking the AMV genome. In the normal chicken DNA only fragments corresponding to the c-*myb* allele were detected (Figure 4A, lanes 1-4). In the transformed lines both the c-*myb* sequences and the fragments expected for the v-*myb* allele were observed (indicated by dots). No new virus integrations within or near the c-*myb* locus in either BM2 or BM2L cells were detected using avian retroviral specific probes (data not shown).

The inability to detect genetic changes in *v-myb* by Southern analysis did not allow us to rule out completely that subtle changes in structure or expression of the gene might be a contributing factor. Nucleotide differences in the DNA-binding domains of various v-*myb* alleles have been shown to have a profound effect on differentiation and gene expression (Introna *et al.*, 1990) and could account for the leukemogenicity of BM2L cells. Therefore, to analyze v-*myb* for point mutations that could contribute to the leukemogenic phenotype of BM2L,

Figure 4. Molecular analysis of BM2 and BM2L cells. (A) Southern blot analysis of the v-*myb* alleles in BM2 and BM2L cells. Genomic DNA from normal bone marrow cells (lane 1-4), BM2 cells (lanes 5-8) and BM2L cells (lanes 9-12) was digested with *Hind*III (lanes 1,5,9), *Bam*HI (lanes 2,6,10), *Kpn*I (lanes 3,7,11) and *Eco*RI (lanes 4,8,12), and hybridized with a *myb* cDNA probe. The v-*myb* DNA fragments observed were the expected size and are indicated by dots. (B) Northern blot analysis of v-*myb* expression in BM2 and BM2L cells. 5 μg of Poly(A) RNA from normal bone marrow cells (lanes 3 and 6), BM2 cells (lanes 1 and 4) and BM2L cells (lanes 2 and 5) were loaded in each lane and hybridized with a *myb* cDNA probe (lanes 1-3) or with a U5LTR probe (lanes 4-6). The bands appearing with the *myb* probe at 7.5 and 2.1 kb represent the full length and subgenomic AMV RNA, respectively. The *myb* probe also detects the 4 kb c-*myb* transcript in normal bone marrow cells (Shen-Ong, 1990). (C) Western blot analysis of the v-Myb protein in BM2 and BM2L cells. Nuclear extracts from 5 X 10^6 BM2 cells (lane 1) or BM2L cells (lane 2) were separated on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose filters. The Myb protein was detected on filters using an anti-Myb monoclonal antibody as described in Chapter 2. Lane 3 represents a blot incubated only with the secondary antibody to show the non-specific background. Arrow shows the position of the 45 kd Myb protein (Klempnauer *et al.*, 1983). Size of molecular mass standards (in kd) is indicated at left.


A
21 –
6.6 –
4.4 –
2.0 –
1 2 3 4 5 6 7 8 9 10 11 12
B
9.5 –
7.5 –
4.4 –
2.4 –
1 2 3 4 5 6
C
1 2 3
v-Myb
– 68
– 43
– 26

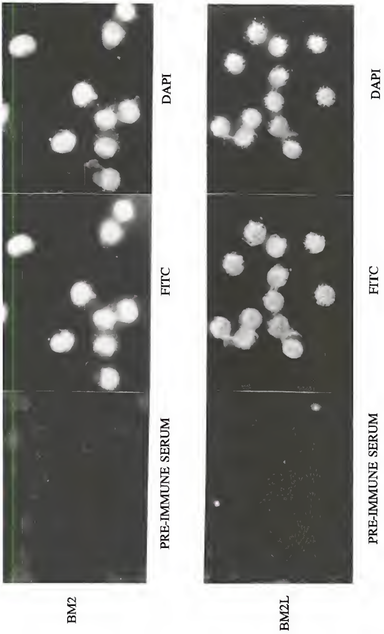
The v-*myb* allele from BM2L cells was amplified, cloned, and sequenced. The 543 bp sequenced at the 5' end of v-*myb* extended from exon 1 into exon 3. These exons encode 181 amino acids of the DNA-binding domain of v-*myb*. At the 3' end, 360 bp containing exons 6 and 7 were sequenced. The results indicated that the v-*myb* allele from BM2L cells was identical in the regions sequenced to the AMV v-*myb* oncogene determined from the BM2 cell line (Klempnauer *et al.*, 1982) (data not shown).

The next step was to analyze levels of expression of c-*myb* and v-*myb* in BM2 and BM2L cells. Figure 4B shows a Northern blot of poly A -selected RNA from normal chick bone marrow, BM2, and BM2L cells, hybridized with a *myb* cDNA exon probe and an avian retrovirus LTR probe (U5L). The *myb* cDNA exon probe detected the 4.0 kb c-*myb* transcript in normal bone marrow cells, as well as the full-length 7.5 kb and subgenomic 2.1 kb AMV RNA (Kim and Baluda, 1988; Shen-Ong, 1990; Schuur and Baluda, 1991) (Figure 4B, lanes 1-3). The retrovirus LTR probe detected both the full-length and subgenomic viral transcripts but not the c-*myb* transcript (Figure 4B, lanes 4-6).

Likewise, western blot analysis revealed that the protein encoded by the v-*myb* alleles was of the expected 45 kD size (Klempnauer *et al.*, 1983). There were no differences between BM2 and BM2L cells in steady-state levels or the size of the v-Myb protein expressed (Figure 4C), nor in its subcellular localization (Figure 5).

The conclusions from these analyses are: (1) BM2L cells are leukemogenic variants of BM2 cells and did not arise from a spontaneous leukemia in the affected bird; (2) new retrovirus infection is not a factor in the emergence of the

Figure 5. Subcellular Localization of v-Myb Protein in BM2 and BM2L cells. Fluorescent micrographs of cells stained with rabbit anti-Myb serum, in which nuclear localization of the antigen was revealed by staining with FITC-labeled goat anti-mouse IgG. Localization of the nuclei was revealed by DAPI staining. Cells stained with preimmune rabbit serum were used as a control.


BM2
DAPI
FITC
PRE-IMMUNE SERUM
BM2L
DAPI
FITC
PRE-IMMUNE SERUM

leukemogenic properties of BM2L cells; and (3) leukemogenesis of BM2L cells in birds is independent of rearrangements in the v-*myb* or c-*myb* alleles (Bottazzi *et al.*, 1993).

Growth Characteristics in Culture Reflect Differences *In Vivo*

Introduction

To facilitate identification of genes involved in AML using the BM2/BM2L model, *in vitro* assays reflecting the *in vivo* phenotypes of the cells were developed. One assay measures the requirements for exogenous growth factors by growth of cells in liquid culture or as colonies in methylcellulose. Both BM2 and BM2L cells grow in culture as nonadherent, immortalized cell lines. Leukemogenic BM2L cells reproducibly form colonies with little requirement for exogenous growth factors, whereas BM2 cells cannot form colonies unless supplemented with high levels of serum growth factors (Bottazzi *et al.*, 1993). The colony assay provided the basis for the identification of altered gene expression in progression of leukemia.

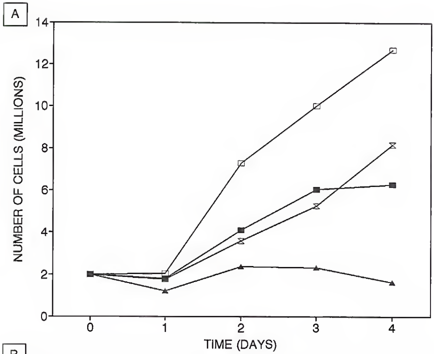
A second assay measures the differentiation potential of cells. In birds, BM2 cells home to the bone marrow and differentiate (Bottazzi*et al.*, 1993). In contrast, BM2L cells do not differentiate, but exit the bone marrow and proliferate. In order to mimic the bone marrow environment in culture, BM2 and BM2L cells were cultivated with a feeder layer of chicken bone marrow cells (BM) or chicken embryo cells (CEC). In addition, differentiation of myeloid cells was induced in culture in the presence of lipopolysaccharide (LPS) and phorbol esters (PMA) without a feeder layer (Symonds *et al.*, 1984).

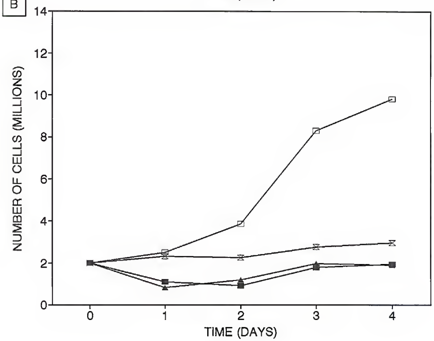
Difference in Growth Requirements

The effect of serum concentration on the growth potential of the two cell lines was examined. After four days in culture with DMEM standard high medium (refer to Chapter 2), BM2 cells grew to a concentration of 6.3 X 10^6 cells/ml (doubling time: 56 h), whereas BM2L cells reached a concentration of 12.7 X 10^6 cells/ml (doubling time: 33 h; Figure 6A). A decrease in serum concentration had a different effect on each cell line. In DMEM standard low medium, BM2L cells continued to proliferate and after four days reached a density of 8.2 X 10^6 cells/ml (Doubling time: 57 h; Figure 6A). In contrast, BM2 cells did not proliferate in decreased serum (Figure 6A). Proliferation resumed when BM2 cells were placed back in DMEM standard high medium (data not shown).

Both cell lines were grown in DMEM depleted high or low medium to determine whether chicken serum and tryptose phosphate broth (TPB) were required for their growth. Results showed that in the absence of both chicken serum and TPB, BM2 cells did not proliferate even when the media was supplemented with high concentrations of newborn calf serum (NBC) (Figure 6B). BM2L cells grew to a density of 3.0 X 10^6 cells/ml of DMEM depleted low medium and 9.9 X 10^6 cells/ml when supplemented with 10% NBC (Figure 6B).

Figure 6. Difference in growth requirements between BM2 and BM2L cells. (A) Growth curves using DMEM standard high and low medium. Growth of BM2 cells (closed triangle and hourglass) and BM2L cells (closed square and open square) in DMEM standard high (hourglass and open square) or standard low medium (closed triangle and closed square). (B) Growth curves using DMEM depleted high and low medium. Growth of BM2 cells and BM2L cells in DMEM depleted high (hourglass and open square) or depleted low medium (closed triangle and closed square). Viability of cells was measured by trypan blue exclusion.


A
14
12
10
8
6
4
2
0
NUMBER OF CELLS (MILLIONS)
0
1
2
3
4
TIME (DAYS)


B
14
12
10
8
6
4
2
0
NUMBER OF CELLS (MILLIONS)
0
1
2
3
4
TIME (DAYS)

These results show that BM2L leukemogenic cells have a diminished requirement for exogenous growth factors and can proliferate in the absence of both chicken serum and TPB (Bottazzi *et al.*, 1993).

Efficiency of Colony Formation

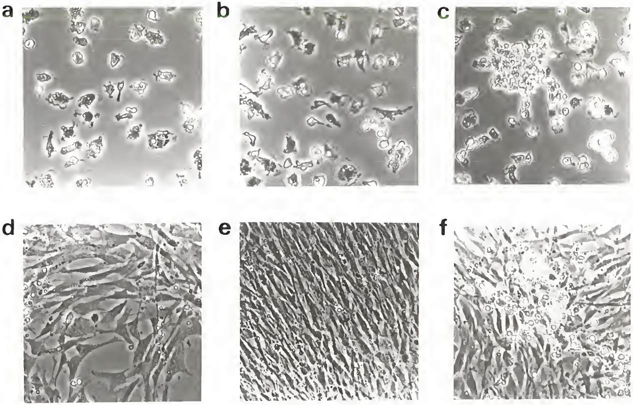
BM2 cells and BM2L cells displayed essentially the same efficiency of colony formation (109 colonies/10^3 cells seeded) in DMEM standard high medium. The cell population comprising a colony was no different in growth from the original population of cells because colonies reseeded in liquid culture had the same doubling time as the parental cells (see Figure 6A). As expected, in DMEM standard low medium BM2L cells formed colonies (67 colonies/10^3 cells seeded), whereas BM2 cells did not.

In conclusion, leukemogenic BM2L cells reproducibly form colonies with little requirement for exogenous growth factors, whereas BM2 cells cannot form colonies unless supplemented with high levels of serum growth factors (Bottazzi *et al.*, 1993).

Adhesion and Differentiation Patterns of BM2 and BM2L

Cocultivation assays *in vitro* were performed in an attempt to mimic the *in vivo* microenvironment. BM2 and BM2L cells were added to freshly seeded bone marrow cells (BM) or a primary culture of chicken embryo cells (CEC) to monitor proliferation, adhesion and differentiation. BM2 cells adhered and differentiated into mature macrophages after three days of cocultivation (Figure 7 b and e). Cell counts

Figure 7. Adhesion and differentiation patterns of BM2 and BM2L cells in the presence of a feeder layer. Phase contrast micrographs taken under an OLYMPUS microscope at 150 X or 125 X magnification for bone marrow or chicken embryo cells, respectively. (a) Normal bone marrow cells (BM); (b) BM cells with BM2 cells; (c) BM cells with BM2L cells; (d) Normal chicken embryo cells (CECs); (e) CECs with BM2 cells; (f) CECs with BM2L cells.


a
b
c
d
e
f

in the supernatant fluid showed that no cells were in suspension and the number of adherent cells corresponded to the sum of the number of feeder cells plus BM2 cells after three days in culture (data not shown). The leukemogenic BM2L cells also had the ability to adhere to the BM or CECs in cell clusters that could not be dissociated from the feeder layers by agitation. However, BM2L cells continued to proliferate and did not differentiate (Figure 7 c and f). After four to five days, BM2L cells detached from the feeder cells and were again present as suspension cells. In addition, and as previously shown by Symonds *et al.* (1984,1986), only BM2 cells were able to differentiate in culture in the presence of LPS and PMA without a feeder layer of stromal cells (Figure 8).

Adhesion of BM2 and BM2L cells was also detected when cells were cultured on a gel containing extracellular matrix components (Matrigel, Biocoat, CRI) even though differentiation of BM2 cells was never observed. Cells were also analyzed by their ability of adhesion and differentiation on individual components of the extracellular matrix. Only BM2 cells were able to adhere to human fibronectin (Biocoat, CRI) (Table 1). Adhesion and differentiation could not be seen using mouse laminin and rat tail collagen type I. These data appear to be inconclusive because there is no evidence of whether the cells express these receptors or would bind to human and mouse components, or whether an effect at higher concentrations could be seen with each matrix component.

These results show that only BM2 cells can be induced to differentiate. It is possible then, that BM2L cells cannot be controlled by the bone marrow

Figure 8. Adhesion and differentiation patterns of BM2 and BM2L cells in the presence of LPS and PMA. Phase contrast micrographs of BM2 and BM2L cells in the presence of LPS and PMA taken under an OLYMPUS microscope at 150X magnification.

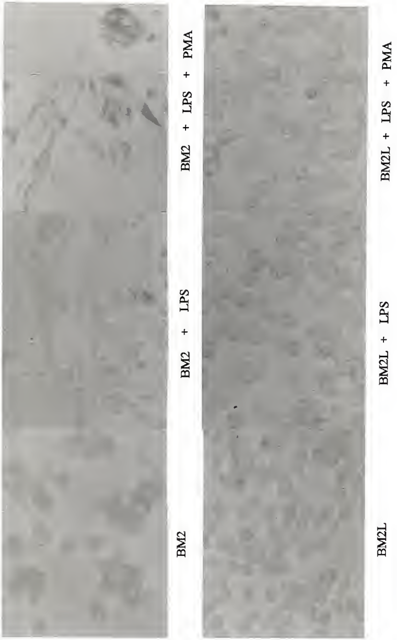

BM2
BM2 + LPS
BM2 + LPS + PMA
BM2L
BM2L + LPS
BM2L + LPS + PMA

TABLE 1.

EFFECT OF EXTRACELLULAR MATRICES ON BM2 AND BM2L CELLS

	BM2 [1]		BM2L [1]	
	ADHESION	DIFFERENTIATION	ADHESION	DIFFERENTIATION
MATRIGEL [2]	+	-	+	-
FIBRONECTIN [3]	+	-	-	-

1 CELLS SEEDED AT 1 X 10^5 PER DISH

2 GEL CONTAINING LAMININ, COLLAGEN TYPE IV, HEPARIN-SULFATE PROTEOGLYCANS, ENTACTIN, TGFB AND OTHER GROWTH FACTORS (BIOCOAT, COLLABORATIVE RESEARCH INC .)

3 HUMAN FIBRONECTIN, MOUSE LAMININ, RAT TAIL COLLAGEN TYPE I (BIOCOAT, COLLABORATIVE RESEARCH INC.)

microenvironment, thus exiting the bone marrow and causing leukemia. In contrast, BM2 cells are induced to differentiate in the bone marrow, which could be the reason why these cells are not leukemogenic. These results established a biological assay to distinguish between BM2 and BM2L cells based on their proliferation and differentiation *in vitro* (Bottazzi *et al.*, 1993). Studies of the homing and differentiation properties of the cell lines *in vivo* were done by Bagnis *et al.* (1993). A summary of the biological differences between BM2 and BM2L cells is shown in Table 2.

TABLE 2.

BIOLOGICAL DIFFERENCES BETWEEN BM2 AND BM2L CELLS

	BM2	BM2L
IN VIVO		
LEUKEMIA	NO	YES
IN VITRO		
IMMORTALIZED	YES	YES
TRANSFORMATION		
GROWTH FACTOR INDEPENDENT	NO	YES
COLONY FORMATION IN LOW SERUM	NO	YES
ADHESION ON BM AND CEC	YES	YES
DIFFERENTIATION ON BM AND CEC	YES	NO
DIFFERENTIATION WITH LPS & PMA	YES	NO

CHAPTER 4

IDENTIFICATION OF GENES INVOLVED IN THE PROGRESSION OF LEUKEMIA

Somatic Cell Hybrids

Introduction

Hybrids between somatic cells provide an approach to analyze gene regulation in eukaryotic cells and to study genetic parameters that govern cell growth, differentiation, and the leukemic process (Ringertz and Savage, 1976). Somatic cell hybrids between BM2 and BM2L cells provide an opportunity to ask if the leukemogenic phenotype is dominant or recessive. One advantage in this system is that intraspecific hybrids in general show greater chromosomal stability than do interspecific hybrids.

Results

In order to have a method for selection of hybrids between the parental cell types, two different drug resistance genes have to be introduced in each cell line. The gene for hygromycin resistance was introduced into BM2 cells by electroporation. BM2 cells carrying the hygromycin resistance gene (BM2Hy) were selected in media containing 250 μg/ml of hygromycin and assayed for growth in culture. This is essential to assure that introduction of the selectable marker does not perturb the phenotype of BM2 cells. BM2L cells carrying the neomycin resistance gene and the lacZ gene (BM2Li6) were obtained from Dr. Claude Bagnis (Bagnis *et al.*, 1993). A drug curve using hygromycin B and G418 was done for each of the cell lines to establish the drug lethal dose (LD50) in liquid culture (Table 3). These results determined the amount of drug needed in the selection after the cells are fused (hygromycin: 250 μg/ml; G418: 1 mg/ml). It has been reported that polyethylene glycol (PEG) can be used to induce cell fusion between mammalian cells (Pontecorvo, 1975; Davidson and Gerald, 1976). Successful hybrids were reported in fusions between cells that grow in monolayers, and by fusions between a monolayer and a suspension cell line (Davidson and Gerald, 1976). BM2Hy cells were fused with BM2Li6 cells using 50% PEG. Hybrids between the cells were selected in media containing both hygromycin and G418, and cloned in methylcellulose containing high serum and both antibiotics (Table 4). As positive controls, unfused BM2Hy and BM2Li6 cells were cloned in the presence of hygromycin and G418, respectively. As negative controls, BM2Hy and BM2Li6 cells were cloned in the presence of G418 and hygromycin, respectively.

After six days in culture, small colonies containing cells with a limited ability to divide, hence, abortive colonies, were visible with Hy/i6 fused cells in the presence of both drugs, as well as with BM2Li6 cells in the presence of hygromycin. After 10 days all cells from these abortive colonies were dead. No colonies were seen when BM2Hy cells were grown in the presence of 1 mg/ml of G418.

TABLE 3. HYGROMYCIN DRUG CURVE

	NUMBER OF CELLS X 10^6 PER ml[a]		
[DRUG]	BM2[c]	BM2L[c]	BM2Li6[c]
NONE	9.11	6.21	24.9
1.00 mg/ml	0[b]	0	0
0.75 mg/ml	0	0	-
0.50 mg/ml	0.3	0	0
0.25 mg/ml	3.3	3.78	0.46
0.10 mg/ml	6.73	5.2	-

[a] All assays done in duplicate with 2 X 10^6 cells per ml

[b] Number of cells after 4 days in the presence of drug.

[c] Cells cultured in the presence of hygromycin

TABLE 4. Colony Formation of Hy/i6 Fused Cells

	Colony Formation		
	G418[1]	Hygromycin[2]	G418+Hygro
BM2Hy cells	neg[3]	pos[4]	neg[5]
BM2Li6 cells	pos	abortive[6]	neg
Hy/i6 cells	ND	ND	abortive

---

[1] 1 mg/ml

[2] 250 μg/ml

[3] No colonies formed after 10 days in culture

[4] Colonies formed after 10 days in culture

[5] Not determined

[6] Small colonies formed that contained cells with limited ability to divide, and containing less than 20 cells

The formation of abortive colonies by BM2Li6 cells in the presence of hygromycin may indicate that the cells in semi-solid media have a different sensitivity to the drug. It is not clear whether the abortive colonies formed by the Hy/i6 cells arose from fused cells or from surviving BM2Li6 cells. As a result, there is no evidence that cell fusion had taken place.

There are several concerns that can be taken in consideration of why fusions may have not been successful. (1) Fusion may be occurring at a very low frequency that is not detected by the assay; (2) if fusion is occurring, the hybrids may be unstable. It is known that among proliferating hybrid cells there is a reduction in chromosome number (Tomita *et al.*, 1992). The chromosomes carrying the drug resistance genes may be lost during replication; (3) the conditions used in these assays may not be optimal for suspension cells.

It has been reported in the literature that due to the unusual nature of the chicken karyotype (78 chromosomes including 18 macrochromosomes, a large number of indistinguishable small or microchromosomes, and the sex chromosomes), it is not practical to use somatic cell hybrids to map chicken genes as used in mammalian systems (Symonds *et al.*, 1986). In view of these results and the fact that a more direct approach would be more useful to identify genes involved in the onset of leukemia, somatic cell fusions were not pursued any further.

Differential Gene Expression

Introduction

The course of normal development as well as the pathological changes that arise in diseases such as leukemias, whether caused by a single gene mutation or a complex of multigene effects, are driven by changes in gene expression.

The differential display method by means of PCR is an approach used in the identification of mRNAs differentially expressed between normal and tumorigenic populations of cells (Liang and Pardee, 1992). For instance, this method detected changes in gene expression of a gene involved in breast cancer metastasis (Liang and Pardee, 1992). Differential display has several technical advantages as compared to subtractive and differential hybridizations. It is less time-consuming and, unlike subtractive hybridization, it allows simultaneous detections of both groups of differentially expressed genes (*i.e.* oncogenes and tumor suppressor genes). This method uses 50 times less mRNA (1 μg) compared to subtractive hybridizations; and the reproducibility from run to run in displaying mRNA patterns with the same RNA sample is greater than 95% (Liang and Pardee, 1992).

Results

The PCR display method identifies mRNAs that are differentially expressed in BM2 and BM2L cells. The objectives were the following: (1) visualize mRNA compositions of BM2 and BM2L cells grown in 1% serum by displaying subsets of mRNAs as short cDNA bands (Figure 9); (2) clone individual bands (Figure 10) and use them as probes on Southern and Northern blots, in order identify the genomic organization and the differential gene expression of each probe (Figure 11 A and B); and (3) cloned products, which are differentially expressed, would be sequenced and compared with sequences in the data bank and then isolated from cDNA or genomic libraries.

To visualize the mRNA compositions from BM2 and BM2L cells, cDNAs were generated using four different 3' oligo-dT primers. Each cDNA was amplified using a combination of each oligo-dT primer and twenty 5' random primers. The display of the mRNA subsets was seen by running the short cDNA bands on four 6% polyacrylamide gels. The gene expression between two isogenic cell lines should be limited to a small number of differences. As expected, results were reproducible (some were done in duplicate) and show a limited number of differences in gene

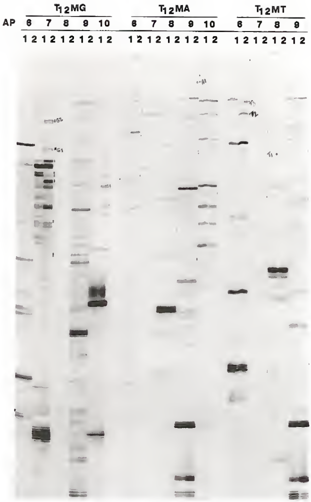


Figure 9. Differential display patterns of RNA from BM2 and BM2L cells. The purified mRNA of BM2 (1) and BM2L (2) cells in 1% serum was reverse transcribed with either T$_{12}$MG, T$_{12}$MA or T$_{12}$MT, where M represents an arbitrary base. Five different combinations of oligo-dT primers and short arbitrary primers (AP-6 to AP-10) were used for the PCR reactions as described in Chapter 2. Differentially expressed bands are represented by small dots or lines hand-drawn adjacent to the lanes.

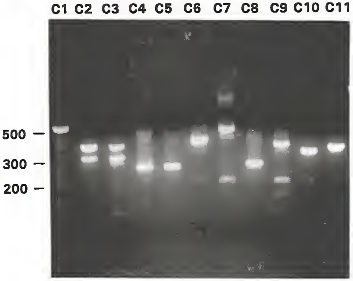
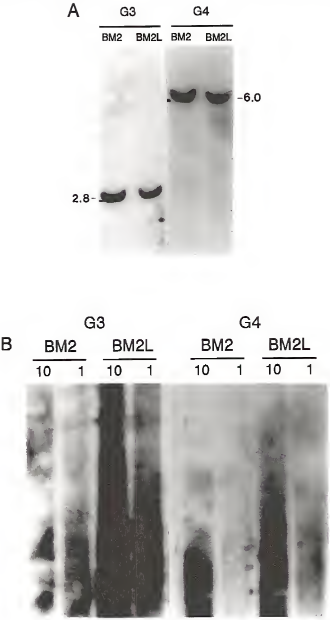


Figure 10. Analysis of differentially expressed cDNA bands. cDNA bands were isolated from a polyacrylamide gel, reamplified with specific primers and visualized on an ethidium bromide-stained gel. Each individual band was cloned for further analysis. Molecular weight standards are shown.

Figure 11. Molecular analysis of two differential display cDNA bands. (A) Southern blot analysis. Genomic DNA from BM2 and BM2L cells was digested with EcoRI and hybridized with G3 (left panel) and G4 (right panel) cDNA probes. The G3 and G4 probes are two representative bands that were excised from the PAGE gel and cloned into pGEM3Z before using them as probes. (B) Northern blot analysis. 5 μg of total RNA from BM2 and BM2L cells, grown with either DMEM standard high (10) or DMEM standard low (1) medium (see chapter 2), were loaded in each lane and hybridized with G3 (left panel) and G4 (right panel) cDNA probes.


A
G3
G4
BM2
BM2L
BM2
BM2L
-6.0
2.8-
B
G3
G4
BM2
BM2L
BM2
BM2L
10
1
10
1
10
1
10
1

expression, which may correspond to the genes involved in the progression of the leukemogenic phenotype (Figure 9). Thirty-three differentially expressed bands were isolated from different gels and cloned for further analysis (Figure 10). Six of the cloned products hybridized to a single band from BM2 genomic DNA cut with *Eco*R1 (Figure 11 A). However, no differentially expressed bands were observed at the mRNA level (Figure 11 B). Sequencing of 4 representative cloned products (A1, G2, T1, and T6) showed that the amplified bands correspond to portions of 3' untranslated regions (data not shown). Twenty of the remaining products were also used as probes to hybridize Northern blots (data not shown).

It is not clear why the cDNA probes only hybridized to Southern blots. One explanation could be that the hybridization conditions were not optimal for DNA-RNA hybridizations. To correct this, hybridizations were carried out using temperature ranges from 45°C to 70°C. Nevertheless, none of the cDNA probes hybridized to mRNA from BM2 or BM2L cells. Another explanation could be that the DNA probes bind nonspecifically to RNA blots. This effect could be partly due to the fact that the probes are short untranslated regions including polyA and other Au stretches (approximately 300-500 bp) (Figure 10). Experiments are in progress to determine if these regions are homologous to any known sequences. In addition, flanking sequences will be cloned to facilitate the identification of these differentially expressed genes.

Insertional Mutagenesis

Introduction

The discovery that insertional mutagenesis plays an important role in oncogenesis led to the use of provirus tagging as a method to identify genes involved in this process. The use of this approach led to the identification of new oncogenes, revealed potential oncogene interactions during the multistep process of oncogenesis, and provided clues about aspects of gene regulation (Tsichlis and Lazo, 1991). Studies on the events involved in the induction of retrovirus-induced tumors are directly relevant to the study of human disease. This is because tumor induction and progression in animals and humans are frequently linked to the activation of the same or related genes (Lazo and Tsichlis, 1990). My strategy involves infection of BM2 cells with a replication competent retrovirus (MAV-2) and selection of cells that continue to grow in 1% serum. This method indirectly identifies genes that contribute to the leukemogenic phenotype in BM2 cells, which may or may not be the same gene(s) altered in BM2L cells.

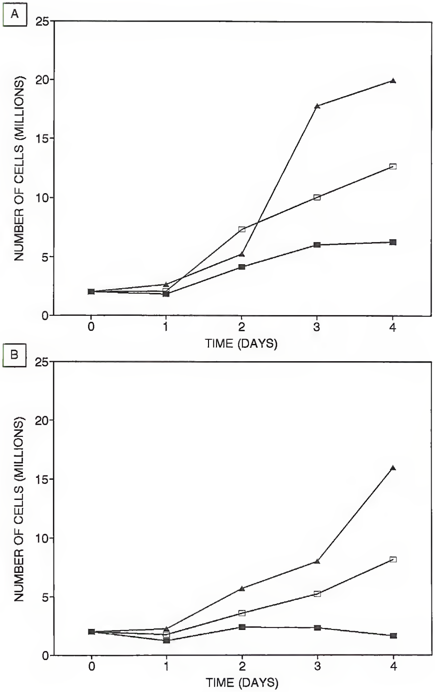
Results

Retroviral infection of BM2 cells was done with the MAV-2 retrovirus. A colony assay was done in 1% serum with cell concentrations of 1 X 10^5 and 5 X 10^4 cells per dish. As controls, BM2 and BM2L cells were seeded at the same time as the BM2-MAV infected cells. After 6 to 10 days colonies formed by BM2-MAV cells were picked and 9 independent clones (M clones) were established. As expected, BM2 cells did not form colonies in 1% serum, whereas BM2L cells did.

The 9 independent clones were checked for MAV integrations by amplification with primers specific for MAV envelope sequences. These specific primers do not amplify *env* sequences from endogenous retroviruses. *env* sequences were amplified from all the cell clones, indicating that a new virus was integrated into the cellular genome.

The effect of serum concentration on the growth characteristics of the BM2-MAV clones was also examined in liquid culture. Figure 12 shows a growth curve of 10.1 cells, a representative clone. After 4 days in culture with DMEM standard high medium, 10.1 cells reached a concentration of 20.0 X 10^6 cells/ml (Figure 12 A). A decrease in serum concentration had a slight effect on the replication capacity of 10.1 cells (16 X 10^6 cells/ml) (Figure 12 B). In addition, the doubling time of 10.1 cells grown in 1% serum increased 2.8-fold (10 hrs in 10% serum versus 28 hrs in 1% serum). All of the BM2-MAV clones showed growth characteristics similar to 10.1 cells. These results confirm that the BM2-MAV clones have acquired the ability to grow in low serum, and illustrates that the method for the *in vitro* selection is highly efficient. Surprisingly, clone 10.1 grows to significantly higher cell densities compared to BM2L cells, both in standard high or low medium. Overall, 10.1 cells have a doubling time that is 3 times faster than BM2L cells in 10% serum and 2 times faster in 1% serum. At least two different explanations would account for the difference in growth characteristics between BM2L and 10.1 cells. First, the gene that

Figure 12. Comparison of growth characteristics among BM2, BM2L and 10.1 cells. (A) Growth curves using DMEM standard high medium. (B) Growth curves using DMEM standard low medium. Growth of BM2 cells is represented by closed squares; growth of BM2L cells is represented by open squares; growth of 10.1 cells is represented by closed triangles. Viability was done by trypan blue exclusion.


A
NUMBER OF CELLS (MILLIONS)
0
5
10
15
20
25
TIME (DAYS)
0
1
2
3
4
B
NUMBER OF CELLS (MILLIONS)
0
5
10
15
20
25
TIME (DAYS)
0
1
2
3
4

is affected by the proviral integration in 10.1 cells is different from the gene mutated in the BM2L cells. Second, if the mutated gene that confers the ability of growing in low serum is the same in BM2L and 10.1 cells, then, the genetic alteration in 10.1 cells has a more drastic effect on the phenotype.

The multistep process of leukemogenesis can be seen in culture by the analysis of cell replication as well as the differentiation capacity of the cells. BM2 cells, which are immortalized, could be considered to be at an early stage in the progression of leukemia. These cells can be induced to differentiate both *in vivo* and *in vitro*, and they require growth factors in order to proliferate. After retroviral integration, BM2 cells acquired an additional genetic alteration that resulted in their ability to grow in low serum. If different genes control the differentiation capacity of cells, the BM2-MAV clones would still have the ability to differentiate. As expected, all of the BM2-MAV clones retained the ability to differentiate when cocultured with a feeder layer of CECs (data not shown). One can hypothesize that additional alterations would be required in order to lose the ability of differentiation, and to become leukemic *in vivo*. Presumably, BM2L cells contain all the genetic alterations that are necessary for a full leukemogenic phenotype (see Chapter 3).

Molecular differences between BM2 cells and the 9 BM2-MAV clones were evaluated. The analysis of the expression of viral messages in the BM2-MAV clones would show the type of retroviral integration in each cell. Figure 13 shows Northern blots of total RNA from three representative BM2-MAV clones hybridized with an avian retrovirus LTR probe (U5L), a MAV *env* probe and a *myb* cDNA exon probe.

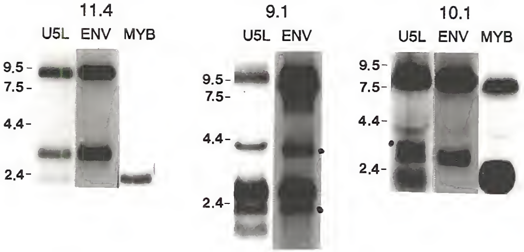


Figure 13. Northern analysis of BM2-MAV clones. 5 μg of total RNA from clones 11.4, 9.1 and 10.1 were loaded in each lane and hybridized with a U5LTR probe (U5L), an *env* probe or a *myb* cDNA probe. Size of molecular weight standards are shown. Novel transcripts for the activated oncogenes are indicated by dots.

The U5L and the retrovirus *env* probes detect the full-length 7.5 kb, and subgenomic 2.7 kb MAV RNA containing the *env* gene. In addition, the U5L probe detects the 2.1 kb AMV RNA which contains the v-*myb* sequences (Kim and Baluda, 1988; Shen-Ong, 1990; Schuur and Baluda, 1991). The *myb* cDNA exon probe detected both the full-length and subgenomic v-*myb* transcript (Figure 13). The transcripts for the activated oncogene in the BM2-MAV clones could begin from either the 5' or the 3' LTR as identified by the U5L probe. These novel transcripts may or may not contain other viral sequences. Transcripts from the 5' LTR and containing viral sequences could be discriminated by using the *env* probe and a leader probe. Leader sequences are diagnostic for transcripts originating from the 5' LTR.

As expected, different types of insertion mutations were observed in the BM2-MAV clones (Table 5). Most of the clones showed enhancer insertions. These clones showed the expected retroviral transcripts but no novel U5 transcripts (Figure 13, Clone 11.4). Surprisingly, clone 11.4 showed a decrease in expression of v-*myb* transcripts compared to the expression observed in BM2 cells (see Figure 4). Clones 9.1 and 9.5 expressed *env* chimeric transcripts that could be explained by insertion with readthrough activation. Clone 10.1 expressed normal retroviral transcripts in addition to a novel U5-positive, *env*-negative transcript.

Proviral integrations in different locations could have as a consequence read-through activation. Clone 10.1 expressed normal retroviral transcripts in addition to a novel U5-positive, *env*-negative transcript. Experiments addressing the specificity of provirus integration have shown that the "apparent" specificity of the integration site

TABLE 5. Mechanisms of Insertion in the BM2-MAV Clones

ENHANCER[1]		PROMOTER[2]	READTHROUGH[3]
3.1	11.1	10.1	9.1
11.3	11.4		9.5
10.3	10.6		

[1] No novel U5-positive transcripts
[2] Novel U5-positive *env*-negative transcript
[3] Novel U5-positive *env*-positive transcripts

in tumors is actually due to the selection of cells carrying these integration events (Tsichlis and Lazo, 1991). Southern analysis using the LTR probe would be able to detect and determine whether viral integrations in the clones occurred in the same or in different locations in the cellular genome. Different patterns of integration would be expected due to the differences previously observed in viral expression. Genomic DNA from BM2 cells and BM2-MAV clones was digested with *Hin*dIII for internal viral fragments and for regions flanking the MAV genome. In BM2 cells, the U5L probe detected fragments corresponding only to AMV and endogenous viruses (Figure 14). In the BM2-MAV clones, AMV, endogenous viruses, and fragments expected from the new integrations of MAV were observed (the latter ones are indicated by dots). The 11.1 and 10.3 clones do not show novel integration bands with *Hin*dIII. Integrations were confirmed by using other restriction enzymes (data not shown). Two clones (11.4 and 9.1) exhibited multiple integration bands. These results are most likely due to high titers of MAV used for infection.

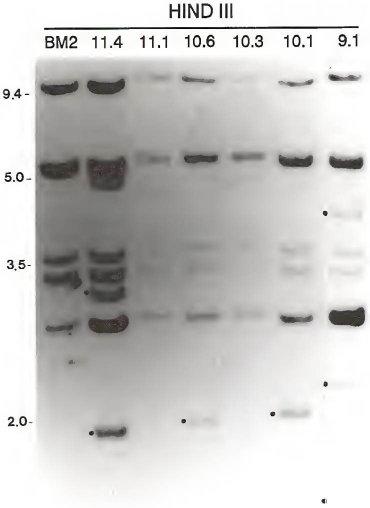


Figure 14. Southern analysis of BM2-MAV clones. Genomic DNA from BM2 and BM2-MAV clones was digested with *Hin*dIII and hybridized with the LTR probe U5L. Fragments corresponding to integration sites by MAV are indicated by dots. Size of molecular weight standards are shown in kb.

Although the results show different patterns of integration, the possibility still exists that the MAV virus integrated in the same gene but at different locations. Retroviral integrations in different areas of the same gene has been previously shown for example, in the *myc* and *myb* genes (Kung *et al.*, 1991). The similar size of the junction fragments in the BM2-MAV clones suggest that MAV may have integrated in the same gene but at different locations (Figure 14 clones 11.4, 10.6, 10.1, and 9.1). This possibility will be addressed below. Further analysis of clone 10.1 was performed because of the very distinct integration pattern at the DNA level and the presence of a novel 3 kb U5-positive, *env*-negative transcript. Additional DNA analysis using different restriction enzymes was done to map in detail the junction fragments of clone 10.1 (Figure 15). The distinct fragment sizes observed in clone 10.1 provided an advantage for the identification of the affected cellular gene.

Several approaches could be used for the identification of the cellular gene at the MAV insertion site in clone 10.1. One could be the production of a cDNA library from 10.1 cells. This strategy would be used to directly identify the novel U5 transcript. The limitation of this approach is that transcripts generated during reverse transcription are generally shorter than 3 kb. Since the transcript of interest is 3 kb in size and the probe available for its detection is located at the extreme 5' end, the screening of the cDNA library would be very difficult.

As an alternative approach, a genomic library was constructed from the 10.1 clone to identify cellular sequences contiguous with the MAV provirus. Genomic

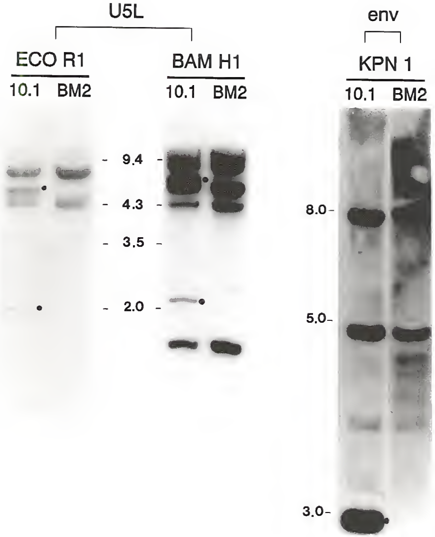


Figure 15. Southern analysis of integrated clone 10.1. Genomic DNA from integrated clone 10.1 was digested with *Eco*RI and *Bam*HI and hybridized with the LTR probe U5L; or digested with *Kpn*I and hybridized with the MAV *env* probe. Fragments corresponding to integration sites by MAV are indicated by dots. Size of molecular weight standards are shown.

DNA from clone 10.1 was partially digested with *Eco*RI and cloned into lambda EMBL4.

Screening of the library was performed by hybridization with the LTR-specific probe (U5L) and the MAV *env* probe (Table 6). A total of 28 lambda clones which were U5 positive, were purified from the library after screening 2 X 10^6 plaques.

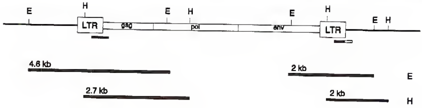
TABLE 6. Genomic Library Screening

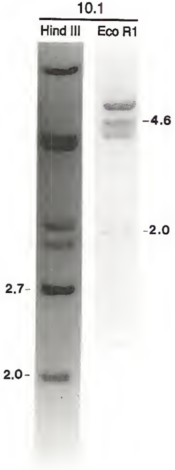
Total Plaques	Primary[1]	Secondary[1]	Tertiary[2]	
			env$^+$ U5$^+$	U5$^+$
2 X 10^6	46	46	5	23
		Total	28	

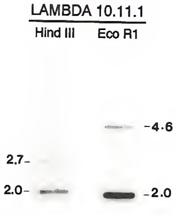
[1] Number of U5 positive plaques isolated from primary and secondary screening
[2] Number of pure plaques after tertiary screening with the *env* and U5 probe

Among the 28 positive lambda clones, only one contained both the 5' and 3' cellular junctions (lambda 10.11.1). A restriction analysis of lambda 10.11.1 compared to 10.1 genomic DNA is shown in Figure 16. This figure depicts the fragments obtained after restriction analysis with *Hin*dIII and *Eco*RI and hybridization with U5L probe. Consistent with the genomic analysis of clone 10.1, lambda DNA digested with *Hin*dIII generated a 2.7 kb internal MAV fragment and a 2.0 kb 3' junction fragment. In addition, *Eco*RI digestions generated a 4.6 kb 5' junction fragment and a 2.0 kb 3' junction fragment (Figure 16).

Figure 16. Southern analysis of lambda clone 10.11.1 using an LTR probe. Lambda DNA from clone 10.11.1 was digested with *Hin*dIII and *Eco*RI and hybridized with the U5L probe (right panel). Results were compared to a genomic analysis using the same enzymes of cell clone 10.1 (left panel). A restriction map of the MAV provirus and the cellular junctions is shown. Numbers represent the size of the expected fragments in kb.


E
H
E
H
E
H
E
H
LTR
gag
pol
env
LTR
4.6 kb
2.7 kb
2 kb
E
2 kb
H


10.1
Hind III
Eco R1
-4.6
-2.0
2.7-
2.0-


LAMBDA 10.11.1
Hind III
Eco R1
-4·6
2.7-
2.0-
-2.0

The 2 kb *Hin*dIII fragment of the 3' junction was subcloned into pGEM3Z (the resulting plasmid was designated pMEB1) (Figure 16). To confirm that the lambda clone 10.11.1 contained an insert that corresponded to MAV and to verify that the 2 kb insert corresponded to the correct junction fragment, sequence analysis was performed (Figure 17). The sequencing data revealed that the junction fragment matched the MAV 3'LTR, which is distinct from endogenous LTRs (Scholl *et al.*, 1983) (Figure 17 A), and continued into non-viral cellular sequences (Figure 17 B). The MAV LTR cannot be distinguished from AMV LTR sequences present in 10.1 cells. To distinguish the MAV insertion site from AMV, the adjacent cellular sequences downstream of MAV were sequenced and compared to the published junction sequences of AMV (Klempnauer *et al.*, 1982; Rushlow *et al.*, 1982). The sequencing data revealed that the cloned 2 kb junction fragment corresponded to the MAV integration site and not the AMV downstream cellular sequences (Figure 17 B).

A *Hin*dIII - *Eco*RI, 1.2 kb, double-stranded DNA fragment (MEB1.2) lacking retroviral sequences, was isolated from plasmid pMEB1. Restriction analysis of DNA from lambda 10.11.1 was done using the 1.2 kb probe to confirm that this probe detected the correct sequences. Lambda DNA digested with *Hin*dIII and hybridized with MEB1.2 detected the 2 kb 3' junction fragment (Figure 18). Furthermore, lambda DNA digested with *Eco*RI and hybridized with MEB1.2 detected a 5 kb EcoRI fragment which corresponded to the downstream *Eco*RI site in the cellular genome (Figure 18). A detailed restriction analysis of lambda 10.11.1 DNA using the U5L and the MEB1.2 probe and additional sequencing data was done to map proviral

Figure 17. Sequence analysis of the MAV 3' LTR and cellular junction from 10.1 cells. (A) Alignment of 3' LTR sequences between MAV and a representative endogenous retrovirus (EV2). (B) Alignment of 3' LTR and cellular junction sequences between MAV and AMV. Dashes represent nucleotide identity; dots were introduced to maximize alignment. The U3, R, and U5 regions of the LTR are indicated above the sequences. H denotes the *Hin*dIII restriction site in the MAV and AMV LTRs.

A

```
                                                         U3
MAV       TGTAGTCTTAATCGTAGG.........TTAACATGTATATTACCAAATAAGGGAATCGCCTGATGCACCAAATAAGGTATTATATGATCCCATTG
EV2       -------AA-TAGAGCCAGAGGCAACC-G--T-GTCTA-AG-----------A--AA--AA--CATT---T--GCTCAT-GG-G...G-GAC-A-

MAV       GTGGTGAAGGAGCGACCTGAGGGCATATGGGCGTTAACAGAACTGTCTGTCCTTGCGTCATTCCTCATC.GGATCATGTACGCGGCAGAGTATGA
EV2       A-AAG------AT---GCA---A-----------AGA-GA-G--A-G-ACGA--ATATA-GCTG-TGC-ACC----AA--AA-............

                                                                                             H
MAV       TTGGATAACAGGATGGCACCATTCATCGTGGCGCATGCTGATTGGTGCACCTAAGGAGTTGTGTAACCCACGAATGTACTTAAGCTTGTAGTTGC
EV2       ...............................................................................................

                   |           R           |                               U5
MAV       TAACAATAAAGTGCCATTCTACCTCTCACCACATTGGTGTGCACCTGGGTTGATGGCCGGACCGTCGATTCCCTGACGACTACGAACACCTGAAT
EV2       ............------T----AT--------------------------A-----A-A---------G-----A----T-G------------

MAV       GAAGCTGAAGGCTTCA
EV2       -----A----------
```

B

```
          H           U3         |          R            |                       U5
AMV       AGCTTGTAGTTGCTAACAATAAAGTGCCATTCTACCTCTCACCACATTGGTGTGCACCTGGGTTGATGGCCGGACCGTCGATTCCCTGACGACTA
MAV       ---------C------------------------------------------------------------------------------------G

                                       |                       CELLULAR JUNCTION
AMV       CGAACACCTGAATGAAGCTGAAGGCTTCAATAGTTGCATCAGTGCAGGTTAGAACAGTGAAGAGACTTAGATTCTGAATTGCTACGTAGGGCTGG
MAV       ---G-----AC-------G----------CGCTG-TA-GA-AGC--CCG-GA--GGAAA--ATAGG-ACCTG-GGT-GGCAT---A-CTACG-AT

AMV       AGATCTTCCTTCTGTGATTGTGCTCAGTTGTTCTCGCTCGTTCTGCATGAGCTTTATGTACTTAGAATATCAAGGAGGAATAAGATGGAATGAT
MAV       GC-AAA-TG-GT----T-AG-AGGA-AG-T-AACAA-AGCC-GG-TT-TTAAA---ATACGAGGATCCT-TT--A
```

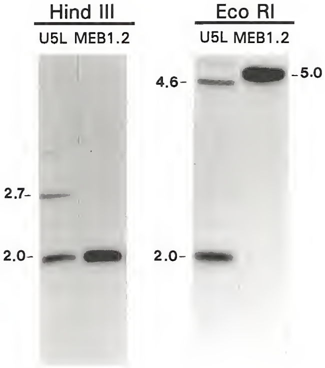
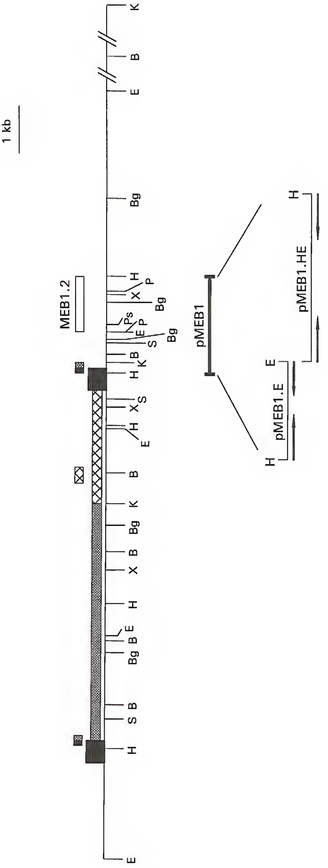


Figure 18. Southern analysis of lambda clone 10.11.1 using a cellular junction probe. Lambda DNA from clone 10.11.1 was digested with *Hind*III and *Eco*RI and hybridized with the MEB1.2 junction probe. Results were compared to the DNA analysis using the U5L probe. Size of expected bands are shown.

Figure 19. Diagram of restriction map of lambda clone 10.11.1. The MAV provirus is represented above the restriction map. Black boxes represent LTRs; stippled bar denotes the *gag* and *pol* genes; Hatched bar denotes the *env* gene; open box represents the MEB1.2 cellular junction probe. The U5L and *env* probes are shown as small boxes above the provirus. The cloned junction fragment (pMEB1) is indicated. An expanded view of pMEB1 shows subcloned fragments pMEB1.E and pMEB1.HE. Arrows designate sequenced regions of each plasmid. Restriction sites detected by sequencing or by restriction analysis are shown. Symbols: E, *Eco*RI; H, *Hin*dIII; S, *Sst*I; B, *Bam*HI; Bg, *Bgl*II; X, *Xba*I; K, *Kpn*I; P, *Pvu*II; Ps, *Pst*I.


1 kb
MEB1.2
pMEB1
pMEB1.E
pMEB1.HE
E
H
S
B
Bg
B
E
H
X
B
Bg
K
B
E
H
X
S
H
K
B
S
Bg
E
P
Ps
Bg
X
P
H
Bg
E
B
K
H
E
E
H

downstream cellular sequences. Figure 19 illustrates a restriction map of lambda clone 10.11.1. In addition, MEB1.2 sequences were used to search for homologous sequences in the Genebank. No match was detected with any known sequences.

The MEB1.2 probe was also used to further analyze the downstream sequences of the proviral integration in the 10.1 integrated cell line and compare the results with the genomic analysis of the parental BM2 cells. Genomic DNA from BM2 cells and clone 10.1 was digested with *Hin*dIII and *Eco*RI and hybridized with MEB1.2. A 3.5 kb *Hin*dIII fragment corresponding to the unrearranged allele was detected in both BM2 cells and the 10.1 clone (Figure 20). In addition, MEB1.2 probe detected the 2 kb junction fragment present in clone 10.1. As previously seen from the lambda DNA analysis, genomic DNA digested with *Eco*RI detected a 5 kb cellular fragment in both BM2 and 10.1 cells (Figure 20).

In order to explore the possibility that the site of integration of MAV in all the selected clones is in the same gene at a similar location, Southern analysis was done using the MEB1.2 probe. Genomic DNA from clones 9.1, 10.6, 11.1, and 9.5 was digested with *Hin*dIII and *Eco*RI and hybridized with the MEB1.2 junction probe. Results show that all the clones contain the 3.5 kb *Hin*dIII cellular fragment but not the 2 kb junction fragment, suggesting that the site of integration in these clones is different from the site found in clone 10.1 (Figure 21). As expected, all clones contained the 5 kb *Eco*RI fragment. A possibility still exists that integrations occurred in the same gene but at different locations. Further analysis of these clones would be necessary to determine the specific integration sites.

Future Directions

Northern analysis of RNA from BM2 and 10.1 cells will determine if the probe MEB1.2 contains any coding sequences. Experiments are in progress to determine whether the 1.2 kb cellular fragment is part of a coding sequence. If it contains no coding sequences, further analysis of the lambda 10.11.1 clone (which contains an insert of approximately 30 kb) will be done to isolate regions containing coding sequences. Coding regions will be sequenced, homology to other genes determined, and used as probes to screen a cDNA library from both normal chicken and 10.1 cDNA libraries. The nature of the mutation(s) in the BM210.1 cells will also be determined by sequencing. The size of the normal and mutated genes will be compared as well as their tissue distribution, to determine if they are myeloid-specific. Furthermore, Northern analysis of the other BM2-MAV clones with a cDNA probe will determine if these cells are mutated in the same gene.

To verify that this gene is implicated in the phenotype observed in 10.1 cells, a construct containing the mutated cDNA will be transfected into BM2 cells. These analyses will ascertain whether the expression of this allele induces the ability to grow in low serum in BM2 cells.

Insertional mutagenesis and the *in vitro* assays developed in this dissertation can be readily adapted for the analysis of the genetic basis of the differentiation pathway.

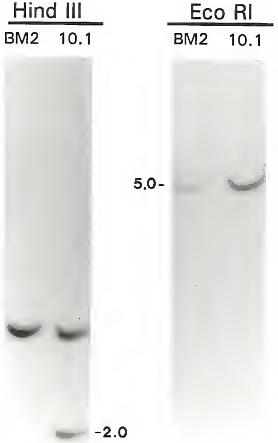


Figure 20. Southern analysis of BM2 and 10.1 using a cellular junction probe. Genomic DNA from BM2 and 10.1 cells was digested with *Hin*dIII and *Eco*RI and hybridized with the MEB1.2 junction probe. Size of expected bands are shown.

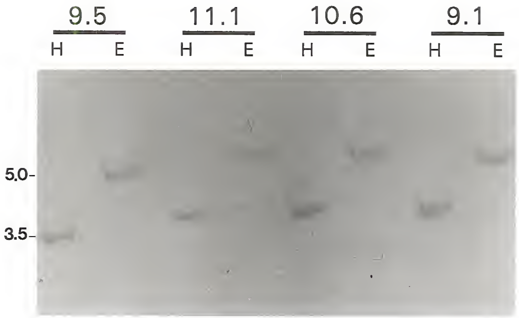


Figure 21. Southern analysis of BM2-MAV clones using a cellular junction probe. Genomic DNA from 9.5, 11.1, 10.6, and 9.1 cells was digested with *Hin*dIII and *Eco*RI and hybridized with the MEB1.2 junction probe. Size of the obtained bands is shown.

CHAPTER 5

DISCUSSION

Introduction

Genes regulated during myeloid differentiation can be classified as those that code for ubiquitously expressed proteins necessary for normal cellular proliferation and survival, for proteins important for cellular differentiation, cellular recognition and cell-cell interactions, and for gene products important for specific functions of the mature monocyte/macrophage (Lubbert *et al.*, 1991).

A variety of proto-oncogenes and suppressor genes have been found to be consistently altered both in structure and function in acute myeloid leukemias (Willman and Whittaker, 1990; Bishop, 1991; Hunter, 1991). Proto-oncogenes may be converted to active oncogenes whenever their structure or function is disrupted by mutation, gene amplification or chromosomal rearrangement. Transformation results when these mutations are dominant, which means that activation of one allele is sufficient. In contrast, tumor suppressor genes function in a recessive manner. To promote leukemia, both normal alleles must be disrupted by deletion or mutation. Genes that have been implicated in the process of leukemia include for example, those that encode growth factors, growth factor receptors, kinases and, transcription factors (Sawyers *et al.*, 1991).

Genes Involved in Signal Transduction are Altered in AML

In vitro studies have established that the proliferation and functional activity of myeloid populations are regulated by a variety of growth factors such as CSFs and interleukins (Metcalf, 1989). In some cases of human AML, mRNA transcripts for CSFs and IL6 have be detected in leukemic blasts. The secretion of these factors induces autonomous proliferation of leukemic cells *in vitro* (Lang and Burgess, 1990; Willman and Whittaker, 1990). The CSF receptor has also been implicated in AML. When this receptor is introduced into myeloid cell lines in culture, high levels of receptor expression may alter growth factor independence and provide the cells with a proliferative advantage (Willman and Whittaker, 1990). Other growth factor receptors implicated in myeloid leukemias are c-*kit*, a tyrosine kinase receptor that is highly related to the CSF receptor, epidermal growth factor receptor (c-*erb*B) and the thyroid hormone receptor (c-*erb*A) (Beug and Graf, 1989; Willman and Whittaker, 1990; Sawyers *et al.*, 1991).

Data from animal models indicate that the enhanced expression of growth factors induce myelodysplasia and myeloproliferative disorders without inducing full leukemia. For example, the avian MH2 retrovirus (containing both v-*myc* and v-*mil*/*raf*) is able to induce macrophages to proliferate independently of cMGF, resulting in autocrine growth of these cells (Beug and Graf, 1989). The ability of v-*mil* to induce production of cMGF is shared with a whole series of cytoplasmic oncogenes, such as the tyrosine kinase oncogenes v-*src*, v-*erb*B, v-*fps*, v-*yes*, and v-*ras* (Beug and Graf, 1989).

In the BM2/BM2L model we can rule out the possibility that an autocrine mechanism is involved in the establishment of growth factor independence. For instance, conditioned media from BM2L cultures containing reduced serum factors, did not enhance the ability of BM2 cells to grow in low serum (Bottazzi *et al.*, unpublished results). These results suggest that the genetic alteration involved in establishment of growth factor independence does not rely on an oncogene that encodes a growth factor which is not normally produced by the cell. Nevertheless, one might speculate that a receptor for growth factor(s) might be involved in this mechanism. Given that the requirement for exogenous growth factor(s) has been eliminated, the pathway must have been activated by another constituent downstream of the growth factor.

There is no particular reason why autonomous growth must involve growth factors or their receptors. When cell surface receptors in myeloid cells are activated, signals must be transmitted from the membrane to the nucleus to cause gene expression. These signals are transduced by the activation of kinases localized inside the cell. In principal, the alteration of a gene encoding a signal transducer protein could bypass completely the normal initial growth factor receptor step. Intracellular kinases, such as the *src* family, the *abl* kinase and the *ras* family of G proteins, have been described to be involved in human leukemias (Liu, 1990; Hunter, 1991; Sawyers *et al.*, 1991). The most frequent molecular abnormality in acute myeloid leukemia, occurring in about 25% of cases, is a mutation in the first exon of the N-*ras* gene (Cline, 1994). Thus, it is possible that an intracellular kinase might be involved in the deregulation of cell proliferation or differentiation observed in BM2L cells.

Integration by retroviruses has been shown as a mechanism that can lead to factor-independence. For instance, in some myeloid- and lymphoid cell lines the constitutive production of the interleukins IL2 and IL3 is caused by proviral insertions (van Lohuizen and Berns, 1990). Therefore, the infection of BM2 cells with a retrovirus could potentially lead to the identification of genes involved in growth factor pathways. Insertional mutagenesis of BM2 cells by infection with the MAV virus conferred upon these cells the ability to grow in medium with 1% serum. Insertional mutagenesis into a normal cellular gene in BM2 cells was sufficient to induce growth factor independence. Mutagenesis of BM2 cells by a retrovirus did recapitulate the phenotype of growth in low serum seen in BM2L cells. However, the phenotype was not identical to the one seen in BM2L cells. All the BM2-MAV clones replicated more rapidly and grew to higher cell densities compared to BM2L cells. Even though insertional mutagenesis of BM2 cells would not necessarily identify the same genetic alteration that occurred in BM2L cells, indirectly it helps to understand what type of genes are involved in growth factor independence. The result from insertional mutagenesis of BM2 cells would rule out the possibility that a tumor suppressor gene is involved in the establishment of growth factor independence. Insertional mutagenesis by retroviruses rarely results in the inactivation of host genes, however, this approach could be used for the identification of activated oncogenes

(Kung *et al.*, 1991). In addition, insertional mutagenesis of BM2 cells would show whether growth factor independence is linked to differentiation arrest. Results suggest that these two phenotypes are not linked and additional alterations are needed to lose the ability of differentiation seen in BM2L cells.

Alteration of Differentiation Genes in AML

Growth and differentiation in normal cells ultimately are controlled by the differential regulation of gene expression through the induction or suppression of transcription (Willman and Whittaker, 1990). A possible mechanism for the arrest of cell differentiation in BM2L cells is the block of transcription of specific myeloid genes that are indispensable for monocyte maturation. In addition, the cloning of translocations and retroviral integration sites from human and animal leukemias has identified genes with homology to known transcription factors, indicating that altered transcription plays a major role in leukemogenesis (Sawyers *et al.*, 1991; Rabbitts, 1994). Cloning of translocation breakpoints and the identification of novel proto-oncogenes in lymphoid leukemias and lymphomas has progressed rapidly, because these translocations often fuse the novel genes with the immunoglobulin (Ig) or T-cell receptor (TCR) sequences. In contrast, the cloning of myeloid breakpoints has been very slow since there are no characterized myeloid-specific genes such as Ig or TCR that can be used as tools (Willman and Whittaker, 1990; Rabbitts, 1994).

Certain transcription factors and nuclear proteins are expressed during myelopoiesis and may be inappropriately expressed in neoplastic myeloid cells. Some genes involved in the regulation of hematopoiesis include, the Fos/Jun family of transcription factors, the Myb transcription factor and tumor suppressor genes such as p53 and RB.

The Fos/Jun family of transcription factors is expressed in normal resting and stimulated myeloid cells, implying that these transcription factors may induce expression of genes that are essential for myeloid differentiation and functional activation (Willman and Whittaker, 1990). Even though normal transcripts have been detected in AML cells, it still remains to be determined if inappropriate expression of these genes promotes abnormal myeloid differentiation or leukemogenesis.

Because v-*myb* induces myeloid leukemias and transforms avian and murine myeloid cells *in vitro*, it has been hypothesized that Myb may play a role in human leukemogenesis. Chromosomal deletions spanning the c-*myb* locus are rarely seen in AML (Willman and Whittaker, 1990). However, recent studies have determined that point mutations localized within the DNA binding domain of the v-Myb protein, specifically in amino acids 91, 106 and 117, can lead to dramatic alterations of gene expression and programs of differentiation in the transformed myeloid cells (Introna, *et al.*, 1990). Expression of the Myb transcription factor, which induces the expression of genes that stimulate proliferation, confers upon BM2 and BM2L cells their uncontrolled proliferation or expansion capacity. In order to rule out the possible role of v-*myb* in the progression of leukemia, the v-*myb* allele in BM2L cells was analyzed for additional genetic alterations that could be involved in the leukemogenic phenotype. I have ruled out the presence of mutations in these regions of v-*myb* in

BM2L cells that could have any involvement in the progression of leukemia. It is possible that mutations in additional genes could cooperate with the v-*myb* oncogene in the generation of the full leukemic phenotype of BM2L cells. Nevertheless, the role of Myb mutations in animal and human leukemias clearly needs to be further examined.

Tumor suppressor genes may also be involved in the progression of both human and animal leukemias. The loss of anti-oncogene function is a common mechanism of the evolution of leukemia and lymphoma (Cline, 1994). For instance, the expression of the p53 gene was shown to be altered in approximately half of cases of AML, and the RB gene was involved in the monocytic variants of AML in about 25% of cases (Cline, 1994). In the BM2/BM2L model, the analysis of differentially expressed genes in BM2 and BM2L cells detected mRNAs expressed in BM2 cells but not in BM2L cells, suggesting the involvement of tumor suppressor genes in the progression from BM2 to BM2L cells.

Oncogene Cooperation and Leukemia

There is little evidence that any one oncogene or tumor suppressor gene has an exclusive function in tumorigenesis (Bishop, 1991). It is even possible that the malignant phenotype results only from the accumulation of multiple genetic mutations. Nevertheless, the distinctive combinations of genetic lesions that appear in different tumors raise the possibility that the abnormalities may collaborate in specific ways in tumorigenesis. For example, the angiogenesis essential to growth of metastases is facilitated by both the production of growth factors encoded by dominant oncogenes and the diminished production of inhibitors of angiogenesis, due to the inactivation of a suppressor gene (Bishop, 1991). It is probable that, as for the metastatic process, oncogene cooperation occurs in other tumor types including leukemia.

Analysis of translocation breakpoints from patients with acute myeloid and lymphoid leukemias implicates growth and differentiation genes in the progression of leukemia (Sawyers *et al.*, 1991). Two animal models demonstrate that genes affecting growth and differentiation can synergize for leukemogenesis. First, the avian erythroblastosis virus (AEV) induces rapid erythroid leukemia in chickens. The genome of this virus encodes two oncogenes, v-*erb*A and v-*erb*B, both of which are required for full transformation. These two genes clearly play a complementary role in avian erythroblastosis (Beug and Graf, 1989). Second, the mouse myelomonocytic leukemia cell line WEHI-3 constitutively expresses IL3 and the homeobox protein Hox2.4, which cooperate in the induction of leukemia (Sawyers *et al.*, 1991).

The specific combinations of oncogenes that were able to cooperate led to the concept that oncogene products that act in the nucleus cooperate best with those that act in the cytoplasm (Hunter, 1991). The generality of this concept is by no means a rule, since oncogenes in the two categories cannot always be interchanged and examples exist where two nuclear or two cytoplasmic oncogenes collaborate (Hunter, 1991). Nevertheless, there is little reason to believe that *in vivo* a single oncogene can generate tumorigenic cells. Although retroviruses carrying single oncogenes give rise to tumors, this occurs largely due to virus spread, which recruits neighboring cells and results in tumorigenesis (Hunter, 1991).

Studies with hematopoietic cells in the chick and mouse systems, showed that combinations of two independent retroviruses can lead to tumorigenesis. For example, in chickens, several oncogenic protein-tyrosine kinases will cooperate with *myb* or *myc* in the transformation of early myeloid cells to render these cells growth factor-independent (Hunter, 1991). One can hypothesize that genetic alterations in at least three genes occurred in BM2L cells. These oncogene products cooperate with each other in the induction of acute myeloid leukemia.

In conclusion, normal cells have multiple independent mechanisms that regulate growth and differentiation, and separate events are needed to override these control mechanisms. Multiple genetic events result in altered gene expression of oncogenes that cooperate in the progression of leukemia.

Conclusions

The appearance of the *in vivo* BM2L leukemogenic variant was clearly a rare event. In the BM2/BM2L model, both the activation of oncogenes and the loss of tumor suppressor genes are involved in the progression of the leukemogenic phenotype. At least three genetic alterations occurred in BM2L cells: immortalization, growth factor independence and loss of differentiation are required for the establishment of myeloid leukemia. For instance, the establishment of growth factor independence in BM2 cells required only one additional alteration. Dominant mutations in growth factor receptors and/or kinases may be involved. These data are consistent with the concept that leukemogenesis is a multistep process where multiple oncogene abnormalities are needed for full expression of the transformed phenotype.

Accumulation of mutations arising in the transformed BM2 cells may have led to changes in genes involved in proliferation and differentiation and hence to the progression of malignancy. The result of these mutations might account for the origination *in vivo* of BM2L cells.

Knowledge of the molecular mechanisms of leukemia by using animal models may have important implications for diagnosis and treatment of human leukemias. For instance, the identification of myeloid-specific genes coding for surface markers could be used to diagnose the presence or absence of leukemic cells after therapy. In addition, molecular probes from these genes may be useful as prognostic indicators of the disease.

A more complete understanding of the genetic mechanisms that occur in the progression of leukemia will also lead to new strategies for circumventing the molecular lesions. One approach is to use gene therapy either to abolish the effects of an abnormal oncogene or to replace a tumor suppressor gene missing in leukemic cells. For instance, the use of antisense RNA has been used in the attempt to block the tyrosine kinase activity of the products of the *bcr-abl* fusion gene in chronic myelocytic leukemia (Cline, 1994). Efforts to insert tumor suppressor genes, such as *Rb* and *p53,* into cancer cells have already begun (Cline, 1994). Ultimately, the main goal is to induce leukemic cells to abandon their malignant phenotype and enter a program of normal cellular differentiation and death. This will be achieved with the understanding of the different cellular signal pathways, their interactions, and their targets. Initial steps have already been successful for the cure of acute leukemias. A good example is the case of acute promyelocytic leukemia and its treatment with retinoic acid derivatives (Cline, 1994).

In summary, normal cells have multiple independent mechanisms that regulate growth and differentiation. Separate events are needed to override these growth control mechanisms in oncogenesis. Multiple genetic events result in altered gene expression of oncogenes that cooperate in the progression of leukemia.

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BIOGRAPHICAL SKETCH

I was born in Genova, Italy, on November 25, 1965. I have lived in Tegucigalpa, Honduras, since I was 8 years old. While in high school I was always interested in science and health related courses and participated in the science fair every year. After high school I entered the microbiology and clinical chemistry program at the University of Honduras. After obtaining my degree I decided to continue my education and learn the latest techniques and advances in medical diagnosis. In August 1989 I entered the Department of Pathology and Laboratory Medicine at the University of Florida. I joined the laboratory of Dr. Maureen Goodenow and collaborated with Drs. Giovannella and Carlo Moscovici in understanding the genetic mechanisms of acute myeloid leukemia. My primary interest continues to be in health sciences, but I also enjoy traveling to exotic places, and enjoying good food and wines. I defended my dissertation in May 1995, got married on July 15 and moved to Miami, Florida, to begin a postdoctoral position with Dr. Assoian in the Department of Cell Biology and Anatomy, University of Miami.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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